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THE EFFECT OF SUB-OPTIMAL TEMPERATURES ON THE GROWTH OF *SALMONELLA TYPHIMURIUM*

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The Effect of Sub-Optimal Temperatures on the Growth of Salmonella typhimurium," submitted by Cayetana L. Hsu, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Salmonella typhimurium was studied at incubation temperatures between the optimum and minimum for growth. The growth pattern at 37, 25, 20, and 15°C was essentially the same, the only difference being a decrease in growth rate with each decrease in temperature. However, at 10°C the cultures exhibited a long lag phase. Microscopic examination of the cultures grown at this temperature showed the presence of long filaments.

The filaments produced at 10°C showed no quantitative difference in macromolecular composition from cells grown at 37°C. Formation of filaments was not markedly affected by the composition of the medium.

When the filaments were transferred from 10°C to 37°C division into normal rods occurred rapidly and was virtually complete within four hours. The presence of metabolic inhibitors at the time of temperature shift did not prevent cell division during the first 30 minutes at 37°C.

The addition of cell extract from a stationary phase culture grown at 37°C to a culture at 10°C greatly reduced the number and length of filamentous cells. Heated cell extract brought about the same response.

Electron micrographs showed that DNA was distributed along the length of the filaments. The cell wall and cell membrane were separated by a periplasmic space whereas this

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INTRODUCTION

"It has been estimated that, if the average daily temperature on the face of the earth as a whole were suddenly raised or lowered by only 20 degrees, all life would perish. This estimate may not be fully correct, but neither is it far wrong, since disastrous consequences to most forms of life would surely follow a sudden change of this sort by seemingly few degrees. Moreover, it bears eloquent witness to the narrow range of temperatures through which life can successfully operate." This quotation from Johnson, Eyring and Polissar (1954) dramatically illustrates the very sensitive relationship between temperature and biological activity. Extensive studies on this relationship have demonstrated major differences between chemical and biological systems and also elucidated many subtle and interesting deviations from the general rules.

In a chemical reaction, the addition of energy to the system in the form of heat will result in an increase in the velocity of the reaction. Provided that the reactants and the products are not heat labile, the increase in velocity of the reaction will continue over a wide range of temperatures.

The relationship between the velocity of a chemical reaction and temperature was studied extensively by Arrhenius who developed the following equation that bears his name:

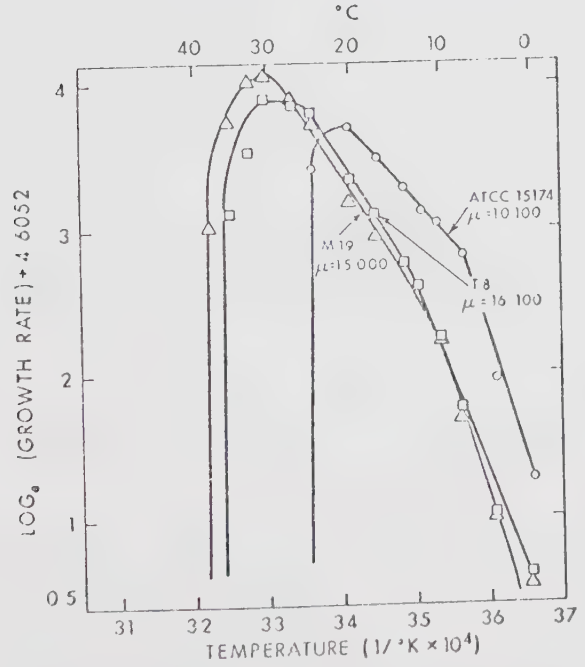
$$v = Ae^{-E/RT}$$

where v represents the observed velocity; R, the gas constant; T, the absolute temperature and E, the activation energy.

From this equation, it is clear that a linear relationship should result when the natural logarithm of the rate of reaction is plotted against the reciprocal of the absolute temperature.

Arrhenius when applying the equation to biological systems substituted μ for E, μ being referred to as the "temperature characteristic." The use of μ instead of E was a recognition of the fact that in biological processes, growth and activity are the result of a complex series of reactions, rather than the result of a specific reaction.

When the Arrhenius equation is applied to biological data, it is apparent that the linear relationship between the rate of reaction and temperature only holds over a narrow range of temperatures. This is illustrated below.



Arrhenius plot of the relationship between growth and temperature of *M. cryophilus* and mesophilic mutants T8 and M19. ○ *M. cryophilus*, □ T8, △ M19.

From Tai, P.C., and H. Jackson (1969)

The deviations from linearity are most likely a manifestation of the complexity of the nature of biological processes. Thus the phenomenon of cellular growth is the result of many hundreds of individual reactions each of which has its own temperature characteristic.

If growth of a particular microorganism is examined over a range of temperature it is usually found to be best in a rather restricted range with the growth rate falling off correspondingly at temperatures above and below this range. At the extremes, growth is not possible and these two limits are referred to as the minimum and maximum growth temperatures. The temperature or narrow range of temperatures at which the organism grows best is known as the optimum temperature. In a living system the optimum temperature may be termed as that point at which metabolic processes function at a maximum rate consonant with the maintenance of the system. In bacteriology, the most common meaning of optimum temperature is the temperature at which the specific growth rate is maximal (Ingraham, 1962). The specific growth rate (k) can be calculated from the equation:

$$k = \frac{2.303\ (\log x_2 - \log x_1)}{t_2 - t_1}$$

in which x_2 and x_1 are any extensive property of the cell at times t_2 and t_1. Most commonly x is a measure of dry weight, absorbance, cell numbers, protein, ribonucleic acid or deoxyribonucleic acid. The value of k, the specific

growth rate, will be the same no matter what component of the cell is measured, provided that the culture is in a state of balanced growth.

Although all microorganisms exhibit minimum, maximum and optimum temperatures the ranges of these temperatures vary considerably from species to species. Thus Escherichia coli has minimum and maximum growth temperatures of approximately 6° C and 46° C respectively whereas for Micrococcus cryophilus the temperatures are - 5° C and 25° C. The temperature range for growth for any particular microorganism is however quite consistent, being within the range of 30 to 40 centigrade degrees.

Temperature response has been used as a basis for the classification of microorganisms into three main groups, namely, psychrophiles, mesophiles and thermophiles. The distinguishing characteristic of the psychrophiles is their ability to grow at the lower temperature extremes. Ingraham and Stokes (1959) defined psychrophiles as microorganisms that grow at 0° C to form macroscopically visible colonies on solid media within two weeks. However, Stokes later (1963) restricted this definition and confined the required formation of visible colonies to one week. Alternative terms have been used for the psychrophiles based on their optimum temperatures for growth hence, those which have an optimum temperature of 20° C or higher are called facultative psychrophiles and those with optimum temperatures lower than 20° C are termed obligate psychrophiles (Farrel and Rose, 1967).

At the upper extremes of temperature, another group of microorganisms is capable of growth at temperatures above 50° C and hence they have been aptly called thermophiles. This group of organisms is further differentiated into strict or obligate thermophiles with optimum temperature for growth between 65° - 70° C but do not grow below 40° - 42° C and the facultative thermophiles with maximum temperature for growth between 50° - 65° C and capable of growth at room temperature. (Farrell and Campbell 1969).

In general, the most favorable temperature for growth of the organism is roughly correlated with the natural habitat, thus most, if not all the bacteria that are pathogenic to or saprophytic on man usually grow best around 37° C and are called mesophiles. Elliot and Heiniger (1965) studied the heat resistance of 14 strains of Salmonella grown on trypticase soy broth in a temperature gradient incubator and found that the maximal growth temperatures fell between 43.2° and 46.7° C and that no strain at 10^6/ml survived 50° C for 48 hours. On the other hand Matches and Liston (1968) found that the minimum temperature that supported growth of S. typhimurium when grown in a similar complex media and growth condition was 6.2° C.

From the above information, it might be asked then what is the physiological or biochemical factor that restricts the growth of microorganisms within certain limits of temperature. Why do some microorganisms tolerate certain temperatures that prove unfavorable for others? Many workers

have searched for an explanation of the phenomenon. Microorganisms in common with all other living cells, contain molecular species including proteins, nucleic acids and lipids, the properties of which are affected by temperature. The heat lability of enzymes is well established and this thermal stability of a number of enzymes and structural proteins has been related to the growth of microorganisms and has been accepted as an important factor that causes the decline in the growth rate of microorganisms when grown at temperatures above the optimum temperature. This concensus has been supported by the work of Evison and Rose (1965) which showed that increasing the temperature of incubation 3° - 5° C above the maximum for growth caused a decrease in the activity of many Tricarboxylic Acid Cycle enzymes and that in a yeast, Candida, the majority of the TCA enzymes were irreversibly inactivated.

Implication of the cytoplasmic membrane as the site of lesion resulting from incubation at temperatures above the optimum has been supported by the work of Siccardi and Shapiro (1971) Inouye and Pardee (1970) on a thermosensitive mutant of E. coli. They found that incubating this organism at 40°C resulted in the alteration of the protein component of the membrane. In addition to this observation, they concluded that this membrane alteration was associated with defective DNA synthesis. Alwood and Russel (1968) and Nozawa et al. (1967) similarly observed membrane damage in Staphylococcus aureus and E. coli respectively when the temperature of incubation was increased. Moreover they also showed that RNA was

degraded and eventually leaked out from the damaged cell. This degradation of ribosomal RNA was found to be due to the increased concentration of ribosome bound ribonuclease at the upper temperature of incubation. Further support for this type of response is the observation of Hagen et al. (1964) on a psychrophilic strain of Serratia which, when incubated 1.5 degree higher than the maximum temperature for growth, lysis occurred rapidly due to the breakdown of certain membrane constitutents. Spratt and Rowbury (1970) isolated a mutant of S. typhimurium with an optimum temperature of 25° C. When this mutant was incubated at 38° C, it increased in cell mass without concurrent DNA synthesis resulting in the production of long filaments and the loss of viability. Similar observations have been reported by Hirota et al. (1968) on E. coli and by Gross et al. (1968) on Bacillus subtilis.

Pace and Campbell(1967) examined the differential heat stability of ribosomes from a variety of psychrophilic, mesophilic and thermophilic microorganisms and found that the thermal stability of ribosomes correlated positively with the maximal growth temperature of the organisms.

Ng et al. (1962) studied the effect of shifting an exponentially growing culture of E. coli ML 30 from 30° C to 10° C and found that the turbidity failed to increase for 4 hours. However, no killing or death resulted from the temperature shift and neither was there an increase in viable cell number. It was later found by Shaw and Ingraham (1967) that during this lag period, there was no net synthesis of

deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein. In view of their result that protein synthesis commenced after four hours of lag and that growth did not recommence till after four and a half hours, it would appear that protein synthesis was necessary for the resumption of growth.

According to Kaempfer et al. (1968), initiation of protein synthesis of E. coli involves an ordered association of the two ribosomal sub-units, 50 S and 30 S, messenger RNA, formyl - methionyl RNA and several protein factors. After the 70 S ribosome completes the translation of the mRNA molecules, it dissociates at some point into sub-units before reinitiating protein synthesis. Friedman et al. (1969) have shown that when the temperature of an exponentially growing culture of E. coli is lowered to 8° C or below, there was an accumulation of ribosomal sub-units. When the temperature was raised above 8° C however, there was a dramatic decrease of ribosomal sub-units. This finding is interpreted as resulting in temperature induced in vivo block in the initiation of protein symthesis but not in the completion of previously initiated polypeptide. Synthesis of RNA and DNA continued even at 7° C.

The above observation could perhaps explain the findings of Goldstein et al. (1964) who found that lowering the temperature of incubation of E. coli to 0° C resulted in the significant over-all decline in protein synthesis which eventually stopped after a few hours. After returning to 37° C, the amino acid incorporation in vivo resumed its

original rate. Friedman et al. (1971) have suggested that the low temperature defect is due to the inactivation of the ribosomes since if cells that have almost ceased incorporating amino acids at 0° C were brought back to 37° C, incorporation started immediately at a rate equal to that in the control cells never subjected to the low temperature treatment. If the temperature was raised only to 10° C the cells recovered their incorporating ability slowly. This temperature shift suggests that at 10° C and higher, the rate of reactivation of the ribosomes is faster than the inactivation reaction, resulting in the reversal of the low temperature defect. They further noted that the accumulation of ribosomal sub-units at low temperature was not dependent on the growth medium. It is likely that an inability to initiate protein synthesis may explain the observation of Ingraham (1958) that cell division in E. coli did not take place below 8° C, a process which naturally requires new proteins. It is perhaps justifiable to assume that for every strain of bacterium, there is a characteristic minimum temperature capable of promoting the initiation of protein synthesis and that this characteristic temperature might also set the minimum temperature for growth.

That low temperature can affect the induced synthesis of enzyme is apparent from the report of Horiuchi and Novick (1961) in which a strain of E. coli was inducible for the synthesis of β- galactosidase at 14° C whereas at 43° C, the cells synthesized the same enzyme in the absence of an

inducer. Synthesis of tryptophanase by another strain of E. coli has been reported to be similarly affected by low temperature (Ng and Gortner, 1963). This decrease in the synthesis of the enzyme has been suggested as the result of the failure of the operator gene to function at low temperature. Marr et al. (1964) have shown that a decrease in the rate of synthesis of β- galactosidase at low temperature is due to the increase in concentration of a repressor with decrease in temperature. O'Donovan and Ingraham (1965) isolated a cold-sensitive mutant of E. coli that has specific requirement for histidine at low temperature. Its requirement for this amino acid was due to feed-back inhibition and the defect appeared to be at the regulatory site of the allosteric protein, PR- ATP pyrophosphorylase, the enzyme that catalyzes the first reaction on the pathway to histidine biosynthesis which was more sensitive to feed-back inhibition than the parent strain. An enzyme that catalyzes the reversible oxidation of D(-)-β-hydroxybutyrate to acetoacetate, β- hydroxybutyric dehydrogenase was found to be severely inactivated at 0° C. Shuster and Duodoroff (1962) found that reactivation of this enzyme was achieved after 6 to 8 minutes at room temperature.

As stated earlier, a change in temperature away from the optimum temperature for growth results in a change in growth rate. Schaecter et al. (1958) studied the effect of medium and temperature on the cell size and composition of S. typhimurium. They have explicitly demonstrated that the

contents of DNA, RNA and protein were influenced by the growth rate afforded by the medium at a given temperature. They further concluded that the size and chemical composition characteristic of a given medium were not influenced by the temperature of cultivation. This statement seems questionable since their experiment was limited to a very narrow range of temperature, and that beyond temperature range they studied (25° - 37° C) it is possible that this generalized statement might not hold true.

Some microorganisms exhibit an increased synthesis of polysaccharides at temperatures lower than the optimum for growth. Tempest and Hunter (1965) reported an increase from 3.3% to 8.9% in the proportion of dry weight accounted for as carbohydrate when the temperature for growth of Aerobacter aerogenes was decreased from 35° to 25° C. Leuconostoc species are induced to produce extracellular dextran at 25° C and this was found by Neely (1960) to be due to the activity of the enzyme, dextransucrase which is inactivated at 30° C. In another case, Ng (1969) working with E. coli found that the cells grown at 10° C contained more carbohydrate than did cells grown at 37° C. Since he demonstrated that at 10° C the growth rate was slower than the respiratory rate, he concluded that the inability of biosynthesis to keep pace with catabolism should result in the diversion of energy from catabolism into forms of storage e.g., glycogen and this is reflected by the higher carbohydrate to protein ratio in cells grown at 10° C

compared to those grown at 37° C.

Several reports have been presented concerning the effect of environmental temperature on fatty acid composition of bacteria; (Ray et al., 1971; Shaw and Ingraham, 1965). However, the actual function of fatty acids in the determination of the minimum growth temperature is not clear. In general the results reveal that low temperature incubation favors the synthesis of unsaturated fatty acids and a decrease in saturated fatty acids. Shaw and Ingraham (1965) rejected the hypothesis that the fatty acid composition of the cells is directly related to the limits of temperature permitting growth. They based their conclusion on their observations that changes in fatty acid composition can also be achieved by varying the composition of the medium and other environmental conditions such as oxygen tension.

Phospholipids are believed to have at least two distinct role in biological membranes. The first role is structural. The lipid component of the membrane is believed to impart much of the uniqueness of the membrane e.g., permeability as well as providing a matrix in which membrane proteins are embedded. Another clearly delineated role for phospholipids is their requirement for the function of certain membrane associated enzymes. Rothfield and Horecker (1964) have demonstrated in S. typhimurium G. - 30, the requirement for the lipid fraction found in the cell envelope in the biosynthesis of lipopolysaccharides and that the activity of the lipid extract in the enzymatic

glucosyl-transferase reactions reside in the phosphatidylethanolamine of the cell envelope. Furthermore, they observed that the fatty acid components of the phosphatidylethanolamine influenced the activity in that, saturated fatty acids had negligible and only slight activity while the highest phospholipid activity was observed when the fatty acid contained at least one double bond or a cyclopropane ring, and conversion of the acyl residues from unsaturated to saturated by catalytic hydrogenation caused complete loss of activity (Rothfield and Romeo, 1971).

Synthesis of bacterial phospholipids and their component fatty acids have been shown to be affected by temperature. Sinensky (1971) noted that the higher the growth temperature of E. coli cultures, the greater the proportion of saturated fatty acids in the bacterial phospholipids. The implication of this observation is best explained by Mavis and Vagelos (1972) and Haest et al. (1972) that the bacteria adjust the fatty acid composition of the phospholipid in response to growth temperature to compensate for the change in permeability (caused by temperature change) by reducing the degree of unsaturation of the phospholipid paraffin chains. Saturated fatty acids tend to render the membrane less fluid while unsaturated fatty acids have opposite effect. Thus, the increase in unsaturation at lower temperature may be viewed as a means of ensuring that the proper degree of membrane fluidity is maintained for the proper functioning of the osmotic barrier.

Okuyama (1969) studied the turnover rate of phospholipids in E. coli after a shift in temperature from 37° to 10° C for a 5 hour lag at the latter temperature and observed that although there was a gradual synthesis of phosphoglycerol, cardiolipin and phosphatidylethanolamine, the synthesis of phosphatidylethanolamine was lower and that the degradation of phosphatidylethanolamine at 10° C which is known to be very stable in normally growing cultures was very much lowered.

From the foregoing literature review it is clear that biological systems exhibit many deviations from a linear response to temperature, particularly as the temperature approaches the minimum and maximum growth temperatures.

Interest in temperature effects on microorganisms has increased tremendously over the last ten years, probably as a result of two factors. Firstly, an understanding of the molecular basis of maximum and minimum growth temperature is of considerable biological significance. Secondly, temperature is probably the most important environmental variable in controlling the growth and activity of microorganisms in food materials and in manufacturing processes based on microorganisms. The more information there is on this subject the greater the possibilities for controlling and optimizing microbial activity. Although the literature on thermal destruction (pasteurization, sterilization) is exhaustive, and although extensive studies have been carried out on the effects of freezing, there is still limited information on

the effects of sub-optimal, non freezing temperatures on the growth and activity of microorganisms. With this in mind, the present study was undertaken to obtain information of the effects of sub-optimal temperatures on Salmonella typhimurium. This organism was chosen because of its great importance as a cause of food poisoning.

MATERIALS AND METHODS

Test Organism and Growth Medium

A strain of Salmonella typhimurium ATCC 13311 (American Type Culture Collection) was used throughout the course of the experiments.

Stock cultures were maintained at 0° C on Trypticase Soy Agar (Baltimore Biological Laboratories, Cockeysville, Maryland, U.S.A.) and sub-cultured at monthly intervals.

The cultural responses of the organism were studied in either a complex medium, Trypticase Soy Broth (TSB) or minimal medium. The minimal medium was the one developed by Davis and Mingioli (1950) and had the following composition:

Glucose	2.0 g	$(NH_4)_2SO_4$	1.0 g
K_2HPO_4	7.0 g	Sodium Citrate	0.5 g
KH_2PO_4	3.0 g	Distilled water	1 liter
$MgSO_4$ .7 H_20	0.1 g		

The pH of the medium was 7.0

Preparation of Inoculum

Trypticase soy broth was prepared and dispensed in 100 ml aliquots into 250 ml Erlenmeyer flasks and sterilized at 121° C for 15 minutes. One flask was inoculated with a loopful of the test organism from a stock culture slope. The inoculated flask was placed in a shaking incubator

(Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) at 37° C and agitated at 200 rpm. When the culture reached the mid-exponential phase of growth, absorbance approximately 1.0 - 1.5, a subculture was transferred to another flask of sterile pretempered medium. At least four further transfers were made in this way before the culture was used as an inoculum in further studies. The purpose of the transfers was to ensure that the growth potential of the cultures was fully expressed and also to obtain cells in a reproducible physiological condition.

Growth Conditions

1000 ml aliquots of medium were dispensed in 2 liter Erlenemeyer flasks fitted with a side arm for sampling and inoculation. A Teflon coated magnetic stirring bar was placed in each flask before sterilization at 121° C for 15 minutes.

After sterilization a flask was placed in a 12" x 12" x 12" plexiglass tank. Water was then circulated throughout the tank in such a way that the water level was above the level of the medium in the flask. Temperature control was achieved by the use of a micro-set thermoregulator (Precision Scientific, Chicago, Illinois, U.S.A.) with a sensitivity of $\pm$ 0.01° C. The thermoregulator was immersed in a water bath and coupled to a heating and refrigeration system, thus maintaining the water bath at the desired temperature. The plexiglass tank plus Erlenemeyer flask was placed on a non-heating magnetic stirrer (Bellco Glass Inc., Vineland, New

Jersey, U.S.A.) to allow for agitation and aeration. Prior to inoculation the flask was allowed to equilibrate at the desired temperature.

The inoculum was prepared as described in the previous section. After 6 transfers from the mid-exponential phase 10 ml of culture was inoculated into the Erlenmeyer flask. This represented a 1% inoculum and normally gave an original cell count of approximately 10^6/ml.

Measurement of Cell Size

Periodically, 0.1 ml of the growing culture was added to 1.0 ml of 0.5 % molten, filtered agar (Difco Laboratories, Detroit, Michigan, U.S.A.). One drop of this cell suspension in agar was then placed on a microscopic slide and a cover slip was pressed firmly onto the drop, expelling all excess material. The preparation was viewed under phase contrast microscopy at a magnification of 700 x using a Leitz Ortholux microscope. Measurement was made using a micrometer eyepiece fitted with vernier scale (Spencer OA, Buffalo, New York, U.S.A.). Size measurement was standardized using a microscope stage micrometer slide (American Optical Co., Buffalo, New York, U.S.A.) with 0 ~ 0.01 mm divisions. 100 cells were chosen at random for length measurements.

To demonstrate division or fragmentation of the very long cells after temperature transfers from 10° C to 37° C. Trypticase Soy Agar was poured evenly onto clean microscope

slides and allowed to cool aseptically by putting the slides in sterile petri dishes. A drop of the culture at 10°C was then spread over the agar and incubated at 37°C. At the desired intervals, smears were then imprinted onto a clean microscopic slide by pressing it against the inoculated agar and the imprint was fixed and stained immediately using the Gram staining procedure. These preparations were then photographed using a Leitz microscope fitted with a camera.

Estimation of Macromolecular Composition

Determination of Carbohydrates

Samples (5ml) were taken in duplicate aseptically at intervals and centrifuged at 9,000 rpm for 10 minutes at 4°C in a Sorvall RC2 B Superspeed Refrigerated Centrifuge (Ivan Sorvall, Norwalk, Connecticut, U.S.A.). The washed pellet was stored at -20°C in screw capped Pyrex test tubes until analysis was to be done. The cells were then suspended in 2N H_2SO_4 to original volume and hydrolyzed at 100°C for 2 hours in an oil bath. The hydrolyzed samples were cooled and analyzed quantitatively for total carbohydrates using the Phenol-Sulfuric Acid method of Dubois et al. (1956).

Phenol in the presence of sulfuric acid reacts with sugar giving a yellow orange color with a definite absorption peak. The intensity of the color is a function of the phenol added. As the amount of phenol is increased, the absorbance increases to a maximum and then falls. Hence the optimum quantity of phenol added to the reaction mixture that gives

the maximum absorbance with a definite concentration of sugar was determined as shown in Table 1.

80% (w/v) phenol was prepared by adding 20 g (20 ml.) of double distilled water to 80 g of phenol (Analytical Grade, Mallinckrodt Chemical Works, St. Louis, Missouri, U.S.A.). This gave a corrrsponding concentration of phenol of 0.83 g/ml.

Sulfuric Acid was reagent grade, 95%, Sp. Gr., 1.84, ACS Certified.

1 ml of the acid hydrolyzed bacterial sample was pipetted into reaction tubes in duplicate. 0.07 ml of 80% phenol solution was added into each sample tube and mixed thoroughly. Then, 5 ml of concentrated sulfuric acid was added using a fast delivery pipette, the stream of the acid being directed against the liquid surface rather than against the side of the tube in order to obtain rapid reaction and good mixing. The reaction mixture was mixed thoroughly in a Vortex mixer (Vortex - Genie, Scientific Industries, Springfield, Massachussetts, U.S.A.) and allowed to cool to 25° - 30° C. The absorbance of the characteristic yellow - orange color was measured in a 1 cm light path at 490 nm in a DBG grating spectrophotometer (Beckman Instruments, Fullerton, California, U.S.A.) against a blank substituting distilled water for sugar solution. The amount of total carbohydrate was determined as glucose against a standard curve previously constructed using glucose (Analytical Reagent, Fischer Scientific, Fairlawn, New Jersey, U.S.A.). See Fig. 1.

TABLE 1

Absorbance of Phenol-Sulfuric Acid and Glucose Mixtures as a Function of Phenol Concentration

Volume Phenol Added	Weight Phenol (µg)	*Absorbance 490 nm
0.01	8.3	.25
0.02	16.6	.37
0.03	24.9	.50
0.04	33.2	.57
0.05	41.5	.60
0.06	49.8	.62
0.07	58.1	.64
0.08	66.4	.63
0.09	74.7	.62
0.10	83.0	.63

* The absorbance is the average of three tests.

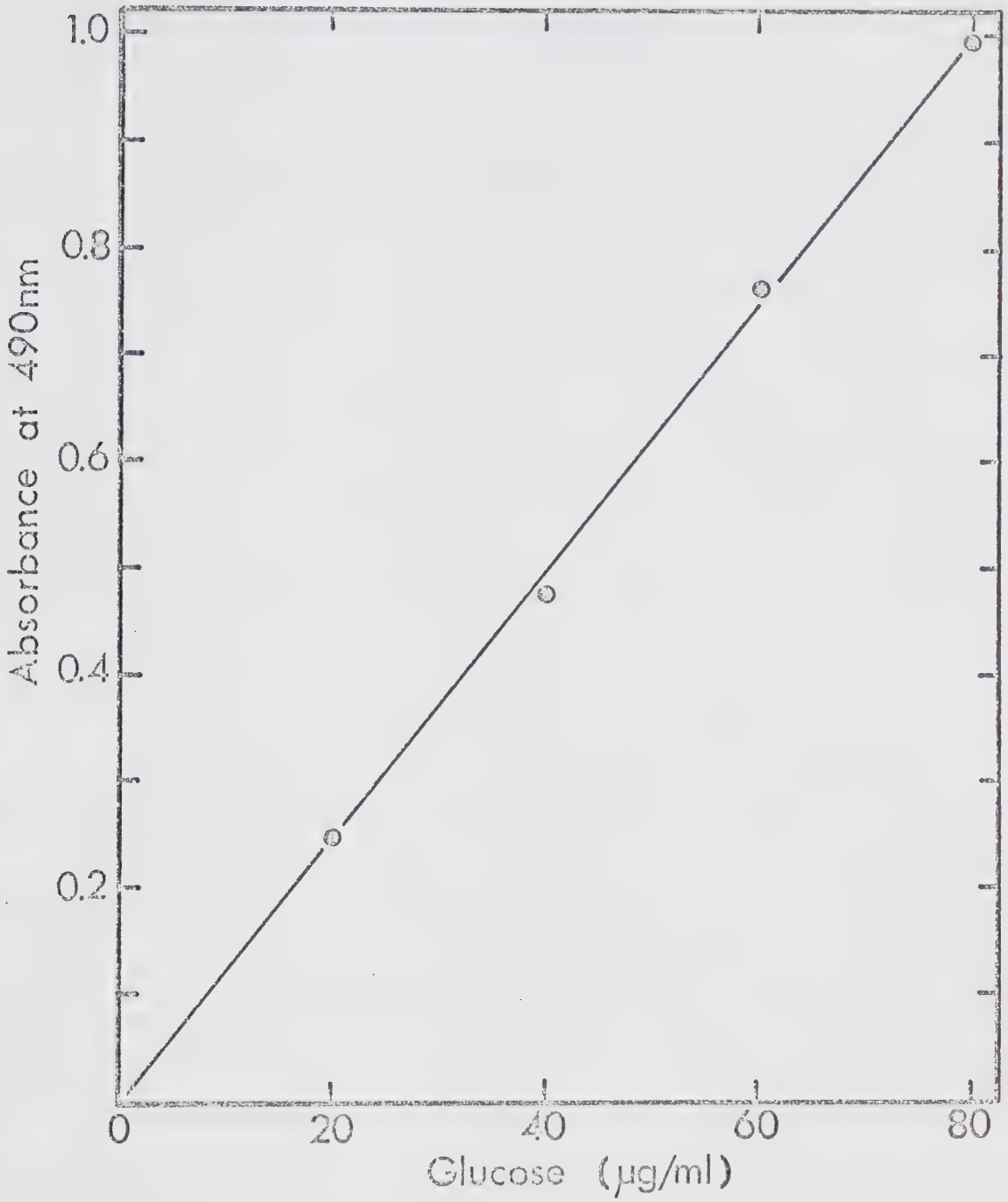


Fig. 1. Standard curve for the determination of carbohydrates

Determination of nucleic acids and protein

General

The procedure for the preparation of biological materials for the quantitative analysis of nucleic acids involves basically first, the removal of low molecular weight compounds such as nucleotides and sugars that might cause interference with the accuracy of estimation. The usual method of removing these interfering substances is by precipitation of the nucleic acids and protein with cold acid, usually trichloroacetic acid (TCA) or perchloric acid (PCA). The temperature and concentration of the acid precipitant is critical as it might cause degradation and loss of RNA. Ogur and Rosen (1950) found that it was necessary to reduce the concentration of perchloric acid to 0.2 N in order to remove acid soluble compounds without running the risk of solubilizing RNA. Munro and Fleck (1967) have observed that 0.2 N perchloric acid also caused maximal precipitation of DNA and protein. They further emphasized the need to maintain the temperature below 4° C as increase in temperature could cause the solubility of RNA and DNA.

For the extraction and separation of nucleic acids, the Schmidt-Thanhausser method (1945) involves the digestion of the cold acid precipitate with alkali which hydrolyzes the RNA and leaves the DNA in a form precipitable upon acidification. Here again, the condition for the separation of nucleic acids is critical. Employment of high temperature

results in a partial degradation of DNA. Fleck and Munro (1962) found that the optimum concentration of alkali for the release of animal tissue RNA to acid soluble form is 0.3 N. These same authors found that at 37° C at least one hour digestion period is required for animal tissues to extract all the RNA into acid soluble form whereas for plants, periods of three hours were found necessary.

Sugiyama et al. (1954) found that there is a considerable range of temperature over which DNA remains stable when incubated in alkali. Essentially, no degradation of DNA to acid soluble products occurs during heating at 50° C for 5 hours.

The separation of RNA and DNA at the end of alkaline digestion involves cooling the alkaline digest to 0° C before acidifying which is essential for the maximum precipitation of DNA. The digest is acidified to a final perchloric acid concentration of 0.2 N and the precipitate is washed of remaining soluble form RNA with 0.2 N perchloric acid. This achieves a virtually complete separation of RNA and DNA.

The preparation of bacterial samples for nucleic acid and protein analyses was basically carried out according to the procedure of Schmidt and Thanhausser. Ten ml of culture was removed at the desired interval and centrifuged at 9,000 rpm for 10 minutes at 4° C. The supernatant was discarded and the pellet was suspended in 0.2 N cold perchloric acid to remove low molecular weight and soluble

compounds. The mixture was mixed in a Vortex mixer and centrifuged at 9,000 rpm for another 10 minutes. The pellet was washed twice with 0.2 M KH_2PO_4/K_2HPO_4 buffer, pH 7.0. The washed pellet was suspended in the same buffer and stored at -20° C for subsequent analysis.

Ribonucleic acid

Ribonucleic acid content of the culture was estimated by the colorimetric method of Dische (1955) using the orcinol color reaction as modified by Lin and Schjeide (1969). This method uses cupric ion as a catalyst in place of the conventional ferric ion.

Cupric ion reagent was prepared by dissolving 0.15 g of $CuCl_2 . 2H_20$ in 100 ml of concentrated hydrochloric acid (C.P. Specific gravity, 1.19) to yield a solution of 8.798 x 10^{-3} F cupric ion.

Orcinol reagent was prepared by dissolving 12.5 g orcinol (Analytical Grade, Fischer Scientific Co., Fairlawn, New Jersey, U.S.A.) in 95% ethyl alcohol to make 25 ml of 50% (w/v) stock solution. For the preparation of 100 ml orcinol reagent that is needed in the analysis 2.0 ml of the stock solution is mixed with 100 ml of cupric ion reagent.

Duplicate 2.0 ml aliquot of the acid soluble RNA sample was pipetted into test tubes. The same volume of orcinol : cupric chloride reagent was added. The reaction tubes were covered with marbles and heated at 100° C in an oil bath for 35 minutes after which they were cooled to room temperature. During heating, the mixture developed a

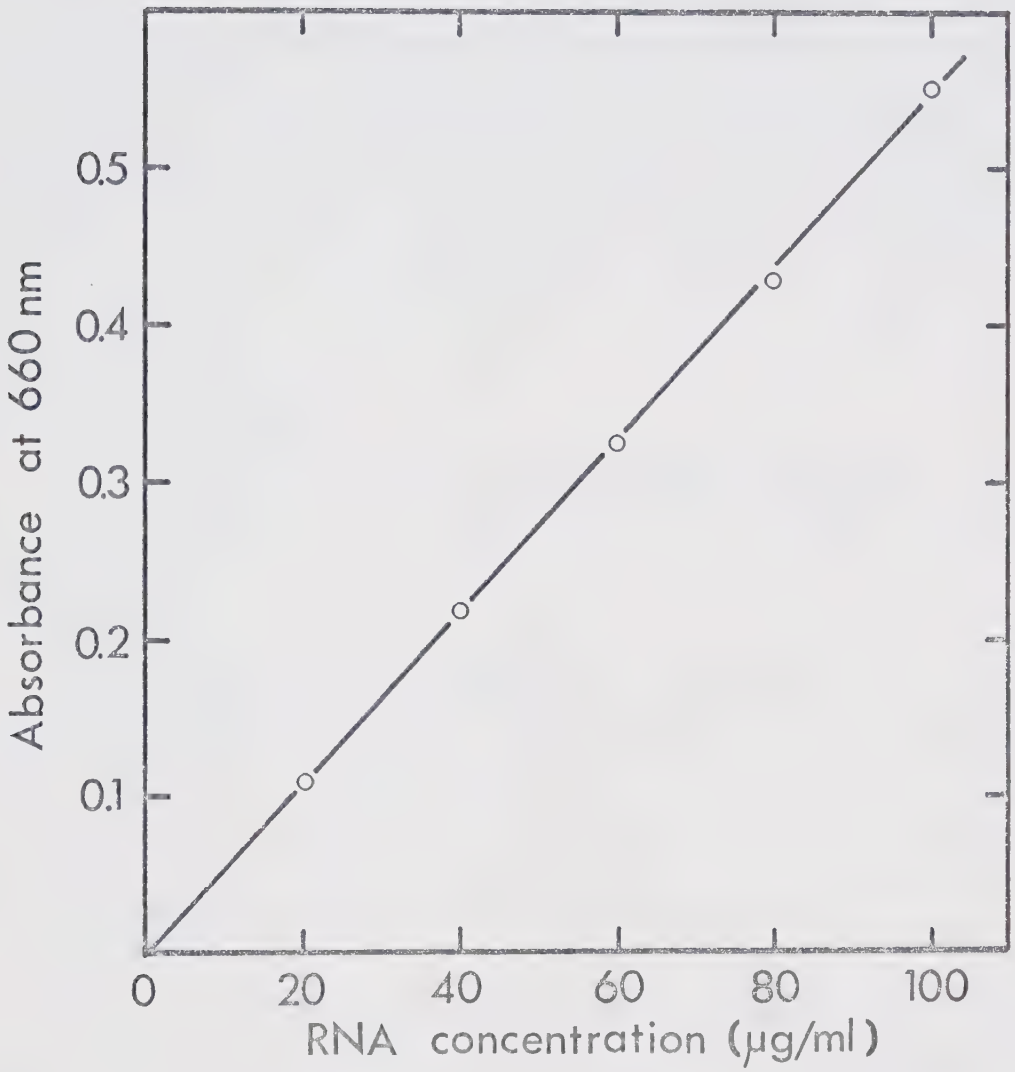


Fig. 2. Standard curve for the determination of RNA

bluish green color, the absorbance of which was measured at 660 nm in a 1 cm light path using a DBG spectrophotometer. The concentration of RNA was determined against a standard curve prepared in the same manner as the samples except for the replacement of the samples with RNA, soluble grade A (Calbiochem Los Angeles, California, U.S.A.) as standard as shown in Fig. 2.

Deoxyribonucleic acid

Deoxyribonucleic acid was determined by the diphenylamine reaction procedure by Dische (1955) as modified by Burton (1956).

The diphenylamine reagent was prepared by dissolving 2.0 g of diphenylamine (Analytical Grade, Fischer Scientific) in 100 ml of glacial acetic acid (C.P.). 1.5 ml of concentrated sulfuric acid was then added and after mixing 0.5 ml of acetaldehyde (16 mg/ml concentration) was added.

The acid insoluble residue from the material after RNA extraction was dissolved in 2.0 ml of 1.0 N perchloric acid. The tubes were covered and incubated at 50° C for 30 minutes. 4.0 ml of diphenylamine reagent was added and the mixture was incubated at 30° C for 18 hours. The blue colored solution was read for absorbance at 600 nm in a DBG spectrophotometer in a 1 cm light path. The concentration of DNA was determined against a standard curve prepared using calf thymus DNA, sodium salt (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) as shown in Fig. 3.

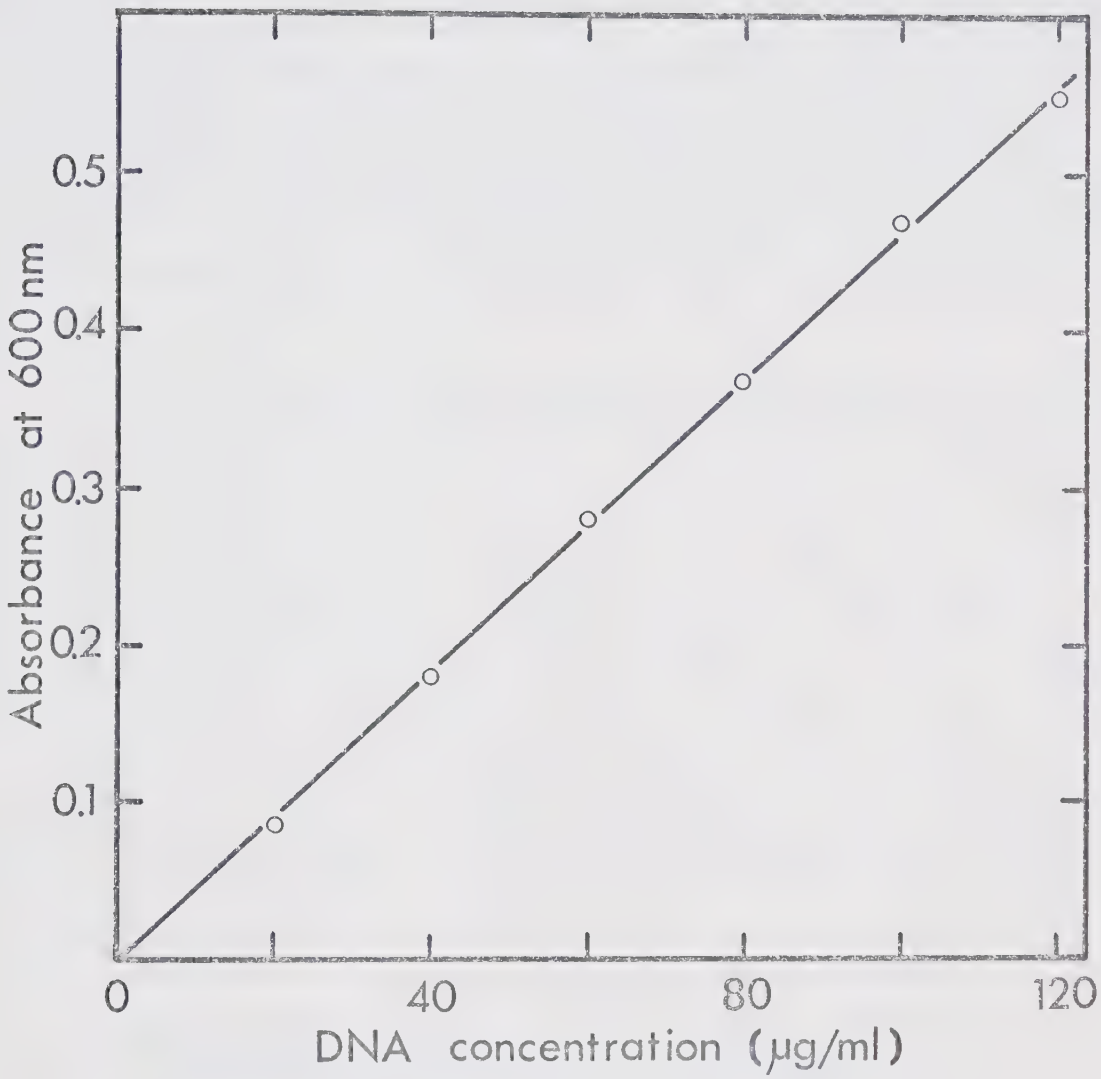


Fig. 3. Standard curve for the determination of DNA

Protein

Protein content was determined by the method of Lowry et al. (1951). 2.0 ml aliquot was taken from the acid washed, phosphate buffer suspended cells prepared for nucleic acid estimations and centrifuged for 10 minutes at 9,000 rpm at 4° C. The pellet was resuspended in 2.0 ml of 1.0 N NaOH and the tubes capped with marbles were incubated at 37° C for 2 hours.

Five ml of the alkali hydrolyzed material was mixed with 2.5 ml of reagent consisting of 2% Na_2CO_3 in 1.0 N NaOH, 1.5% $CuSO_4$. 5 H_20 and 2% Sodium tartrate (100: 1: 1) left aside for 10 minutes after which 0.25 ml of 1.0 N Folin Ciocalteau reagent was added. The mixture was left to react for 30 minutes at room temperature after which the absorbance was measured at 500 nm. Protein content was then determined against a standard curve using bovine serum albumin (Pentex Incorporated, Kankakee, Illinois, U.S.A.) as shown in Fig. 4.

Effects of Inhibitors of Cell Wall, Protein and DNA Synthesis on Cell Division

The bacterial strain under investigation was allowed to grow at 10° C for three days after which time filaments had formed. Five ml aliquots were transferred in duplicate into 250 ml flasks containing sterile trypticase soy broth. Each flask contained a specific inhibitor e.g. Pencillin G, 10 µg/ml (Sigma Chemical Co., St. Louis, Missouri) D - cycloserine, 50 µg/ml, (Sigma Chemical Co.) Chloramphenicol

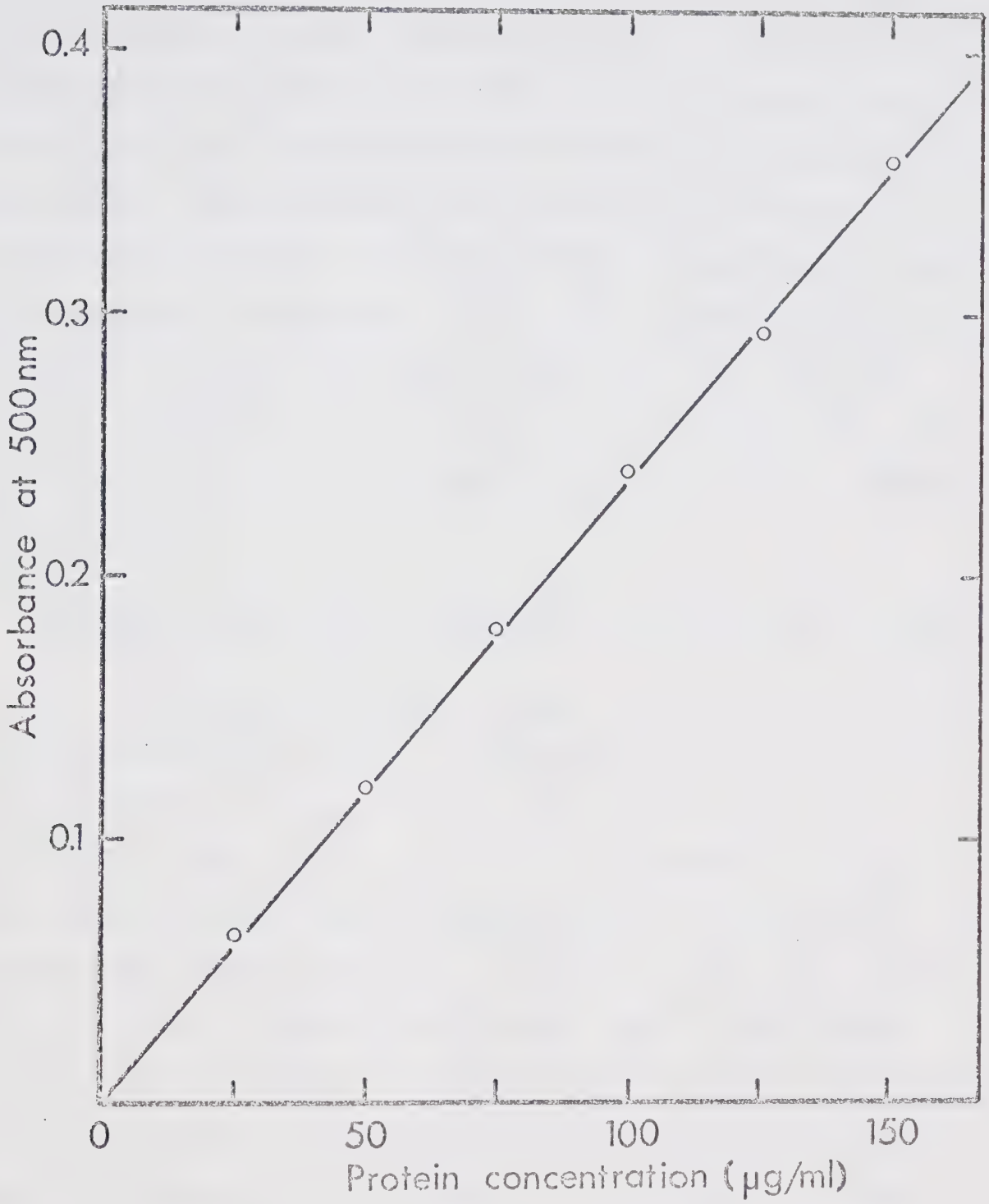


Fig. 4. Standard curve for the determination of protein

10 µg/ml (Sigma Chemical Co.) and nalidixic Acid, 10 µg/ml (Calbiochem, San Diego, California, U.S.A.). These media, including a duplicate control which did not contain any inhibitor, were pretempered at 37° C before the inoculum was added. The ability of the filaments to divide into normal rods after the cultures have been transferred to the optimum growth temperature of 37° C was determined by the increase of cells with colony forming ability. This was done by plating 1.0 ml of the culture into tryptic soy agar in triplicate at precise intervals from the time of transfer for 4 hours.

The Effect of Bacterial Extract on Cell Division at 10° C

Bacterial extract was prepared from 400 ml volume of culture grown for 24 hours in trypticase soy broth at 37° C with aeration. This volume was apportioned into 40 ml centrifuge tubes and the cells were sedimented by centrifugation at 6,000 x g for 10 minutes at 4° C in an RC2' B' Sorvall High Speed Centrifuge. The cell pellet was washed twice with 0.1 M KH_2PO_4/K_2HPO_4 buffer pH 6.8. The washed pellet was resuspended in 10 ml of the same buffer and the cells broken down by sonification for 10 minutes using a sonifier equipped with a microtip (No. 7 setting, Branson Model S - 75, Branson Instrument Inc., Stamford, Connecticut, U.S.A.). The sonicated cell suspension was again centrifuged at 13,000 rpm for 30 minutes at 4° C. The supernatant fraction was sterilized by passing the fluid through a sterile

Millipore filter unit using a filter paper of 0.45 μ porosity (Millipore Filter Corporation, Bedford, Mass., U.S.A.). 1.0 ml of the resultant extract was added to each of four 100 ml sterile flasks of trypticase soy broth, two of which were then heated at 100°C for 10 minutes in an oil bath and the other two unheated. The four flasks, together with a duplicate of extract-free medium as a control were pretempered at 10°C and inoculated with 1% of exponentially growing culture. These were then incubated at 10°C in a metabolite shaker bath (New Brunswick Scientific) and aerated by circular agitation at 200 rpm. The ability of the extracts to promote cell division was determined by the differential increase in colony forming cells as compared to the control cultures. Colony counts were determined by plating 1.0 ml of each culture in tryptic soy agar as done previously.

Preparation of Cells for Electron Microscopy

The cells obtained from growing cultures incubated at 37°C and 10°C were centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge and washed once with 2% sodium acetate.The cells were then fixed according to the method of Kellenberger et al. (1958) as follows: The pellet of cells was suspended in 1 ml of 1% osmium tetroxide (Fisher Scientific) in acetate - veronal buffer, pH 6.1. 0.1 ml of tryptone

medium was added and then left in the dark overnight at room temperature. The fixed cells were washed with acetate - veronal buffer and the suspended in 2% Noble agar and after solidification, the agar suspended pellet was then cut into small blocks. These were then treated for 2 hrs in 0.05% uranyl acetate solution in the buffer. The blocks were dehydrated in a series of acetone solutions of varying concentration (25%, 50%, 75% each for 15 minutes; 90% for 30 minutes; 100%, 2 times for 30 minutes). The dehydrated blocks were put in propylene oxide for 30 minutes and then embedded according to the method of Luft (1961).using Epon as the embedding resin. The Epon was formulated from Epon 812 (Shell Co., San Francisco, California, U.S.A) and methyl nadic anhydride (MNA) (National Aniline Division of Allied Chemical and Dye Corporation, New York City, U.S.A.) in a 1 : 1 ratio. The cubes were left in a mixture of Epon + MNA : propylene oxide, 1 : 1 , overnight, unstoppered and then transferred to fresh Epon in capsules and polymerized at 35^{o}C for 1 day, at 45^{o}C for 1 day and at 60^{o}C for several days. After polymerization the embedded preparations were cut with a Reichert microtome Model OM U - 2 using a diamond knife. Sections were stained with 25% uranyl acetate in methanol for 4 - 6 minutes, washed with 5% methanol followed with distilled water and re-stained with 0.5% lead citrate in dilute sodium hydroxide for 4 - 6 minutes and washed with distilled water. The preparations were examined using a Philips EM 300 electrone microscope.

RESULTS

Growth

Preliminary experiments were carried out to measure the growth response of S. typhimurium in TSB at various temperature from 10° to 30° C. The results are shown in Fig. 5. The data are based on duplicate determination on individual samples from at least two complete runs. The behavior of the cultures at all temperatures was essentially the same, with the exception of 10° C. At this temperature, the growth curve deviates somewhat from a typical exponential form. During incubation at 10° C the cell mass which was measured in optical density units at 450 nm did not increase proportionally with the increase in cell number. The specific growth rate determined by cell mass was 0.048 whereas, the equivalent in cell number was 0.022. In view of this it was decided to look in more detail at the temperature response of the test organism at 37° C and 10° C.

Cells were grown at 37° C in TSB and then transferred to fresh medium at 37° and 10° C, the inoculum being 1% by volume. Growth response was measured as colony count and absorbance at 450 nm. The results are shown in Figs. 6 and 7. It can be seen that in the culture grown at 37° C the colony count and absorbance are parallel, whereas at 10° C, the absorbance shows a greater increase than the colony count. As the absorbance and colony count at 10° C do not

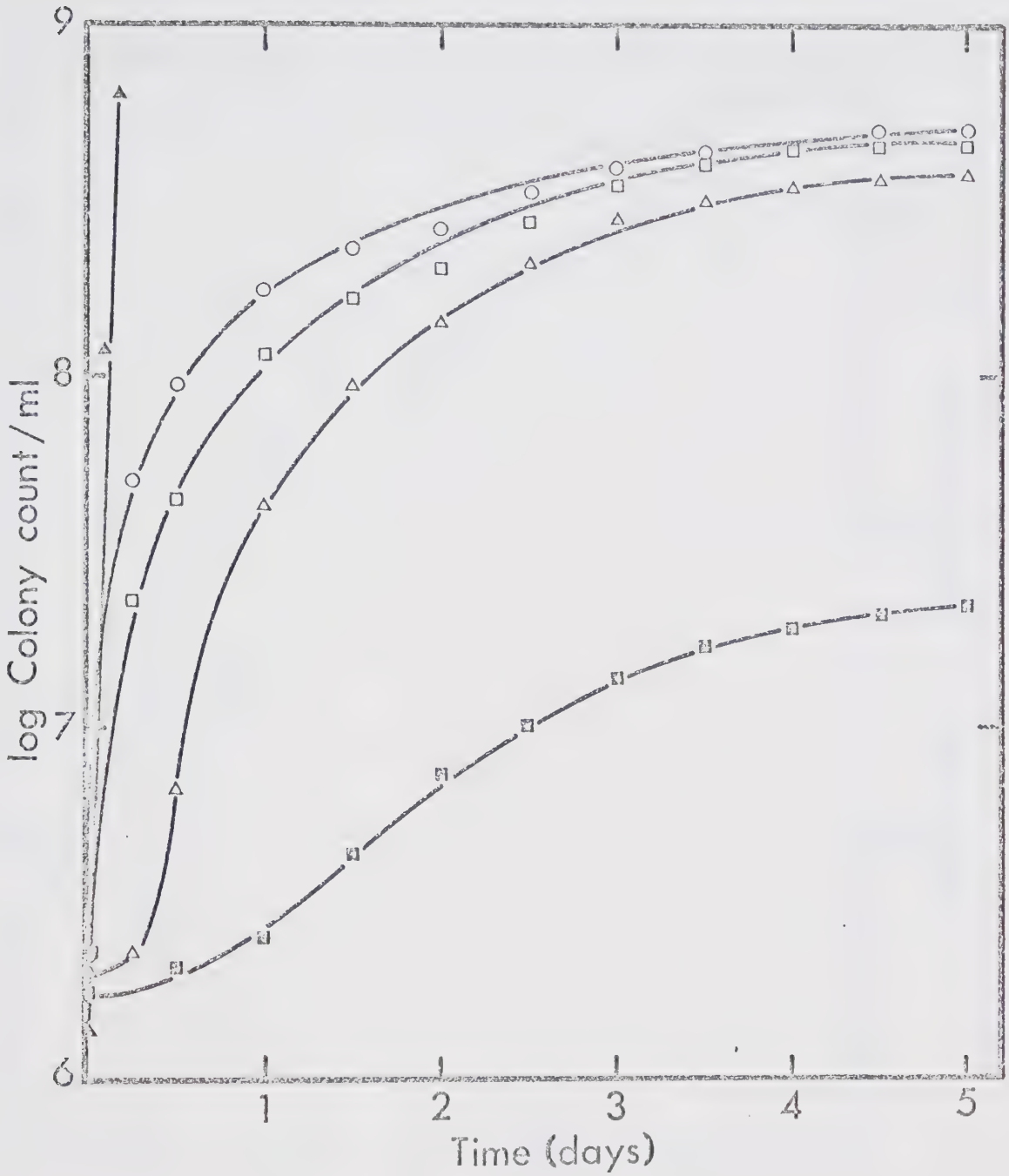


Fig. 5. Growth curve of S. typhimurium grown in trypticase soy broth at different temperatures. △- 37° C; ○ - 25° C. □ - 20° C; △- 15° C and ▯- 10° C.

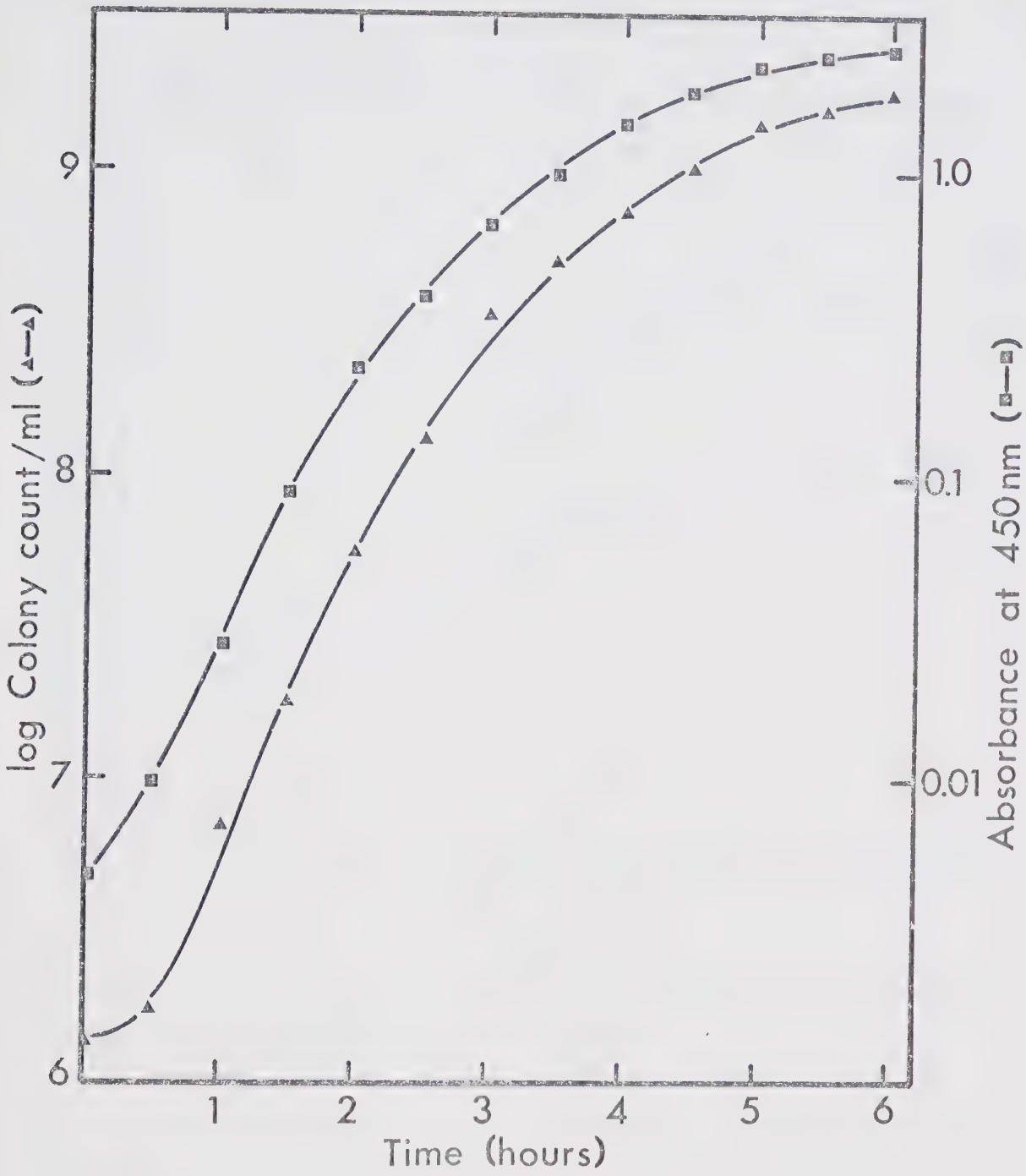


Fig. 6. Increase in colony count and absorbance of a culture of S. typhimurium at 37° C.

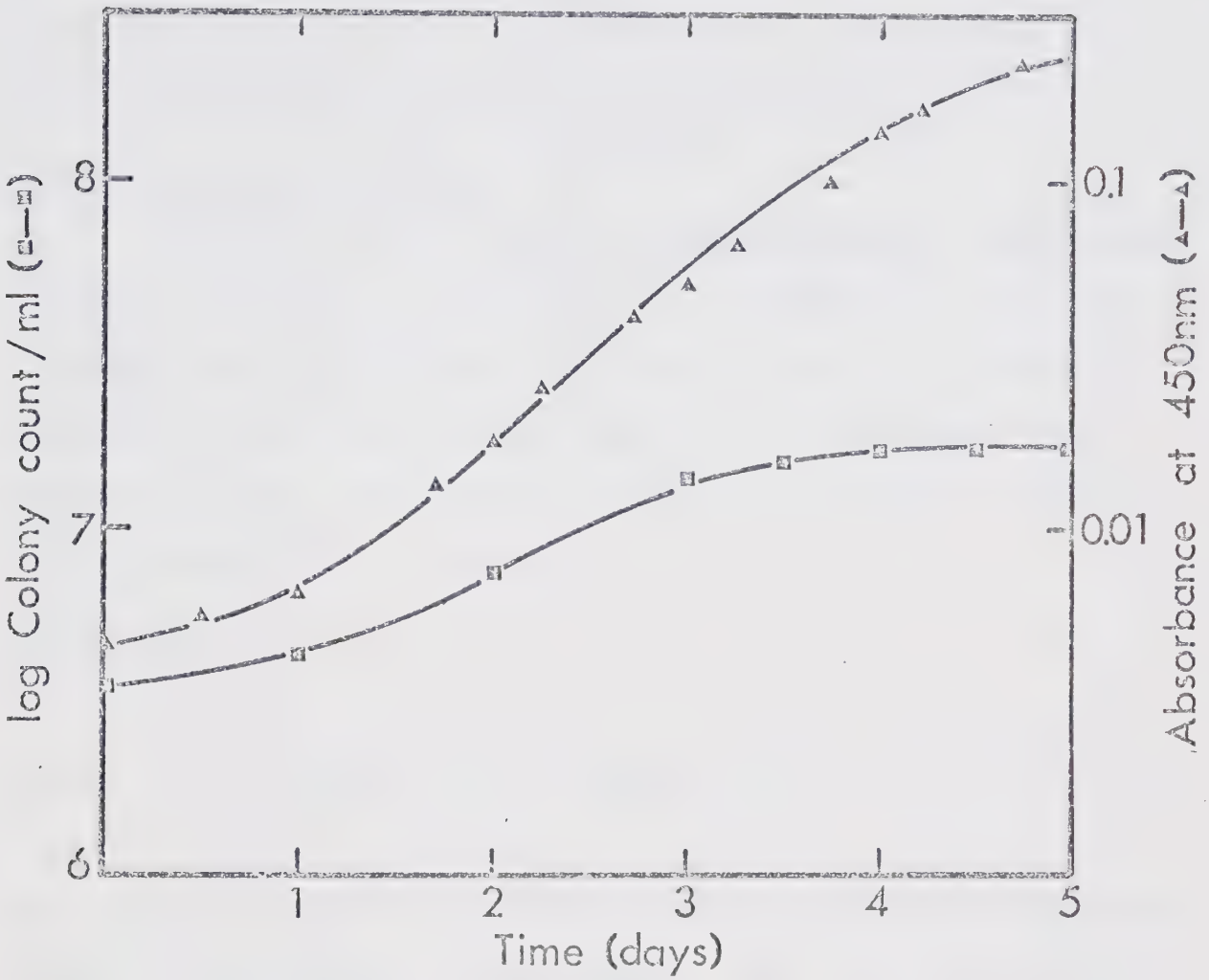


Fig. 7. Increase in colony count and absorbance of a culture of S. typhimurium at 10° C.

show the same proportional increases, the result might suggest that the culture is in a state of unbalanced growth. If so, this would be reflected in differences in the proportion of macromolecules at 10° C compared to 37° C.

Macromolecular Composition

Results of the analysis of macromolecular composition are shown in Table 2. The results are presented as the ratio of RNA/Protein and RNA/DNA. It can be seen that no marked differences were apparent during growth at 10° C and 37° C. Determination on carbohydrate content also showed no differences between the two samples.

Morphology

Microscopic examination of cultures growing at 10°C showed that after a lag of approximately 24 hours the cells showed evidence of undergoing cell division. However after one round of division the cells were seen to elongate without cell division actually taking place. This could account for the non-linear relationship between absorbance and colony count (see Fig. 7). Since cell division at 10° C is a very slow process, the extent of division the cells were undergoing could readily be observed under phase contrast microscopy. As it took approximately 24 hours lag before the cells went through a burst of cell division activity, it was possible to determine after approximately the same time interval if the cells were undergoing further cell division. The number of cells showing evidence of going

TABLE 2

The Effect of Temperature on the Macromolecules of S. typhimurium Grown on Trypticase Soy Broth

Temperature	Optical density, 450nm	RNA/ Protein	DNA/ Protein	Carbohydrate/ Protein
37° C	0.01	0.71	0.22	0.80
	0.10	0.76	0.20	0.88
10° C	0.01	0.66	0.23	0.88
	0.10	0.71	0.19	0.91

Temperature	Optical density, 450nm	RNA/ DNA	Protein/ DNA	Carbohydrate/ DNA
37° C	0.01	3.2	4.5	3.6
	0.10	3.8	5.0	4.5
10° C	0.01	2.9	4.3	3.7
	0.10	3.6	5.2	4.7

through the division process, as evidenced by the presence of constrictions along the length of the filaments or cells and the percentage of such dividing cells were determined. The results appear in Fig. 8. It would appear that after a burst of cell division at approximately 24 hours, the percentage of dividing cells declined progressively during prolonged incubation. This is supported by the results shown in Fig. 9, where it can be seen that with continued incubation, the average cell length of the bacteria increased progressively. Although some filaments could increase in length to as much as 150 times that of the regular rods, they do vary in length so that, the average length of 100 filaments measured at random was 37 μ after a five day period of incubation.

The observation on the effect of transferring the culture to low temperature parallels the results of Ng et al. (1962) which showed that growth at low temperature alters or damages the cells and results in a decreased growth rate. However, this present experiment has succeeded in showing a more realistic physiological evidence of that incurred cell damage by the resultant inhibition of cell division and eventual growth of the rods into filaments.

Morphological observations were also made at 8° C and 12° C in complex media and at 15° C in minimal medium. At 8° C, the cells grew into filaments but were of shorter length than those attained at 10° C. At 12° C there were some filamentous growth although the normal rods were

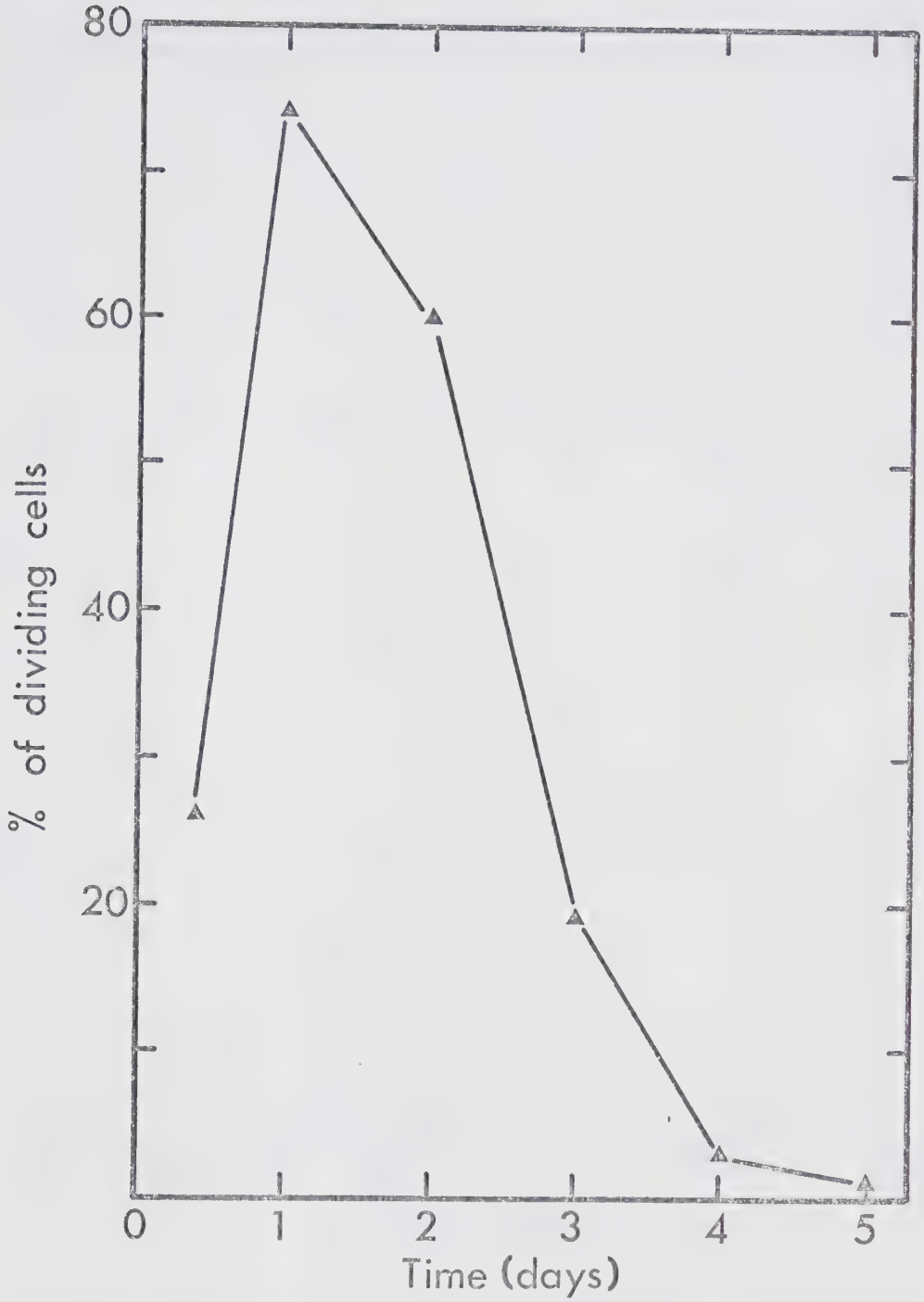


Fig. 8. Loss of cell division potential in S. typhimurium during incubation at 10° C.

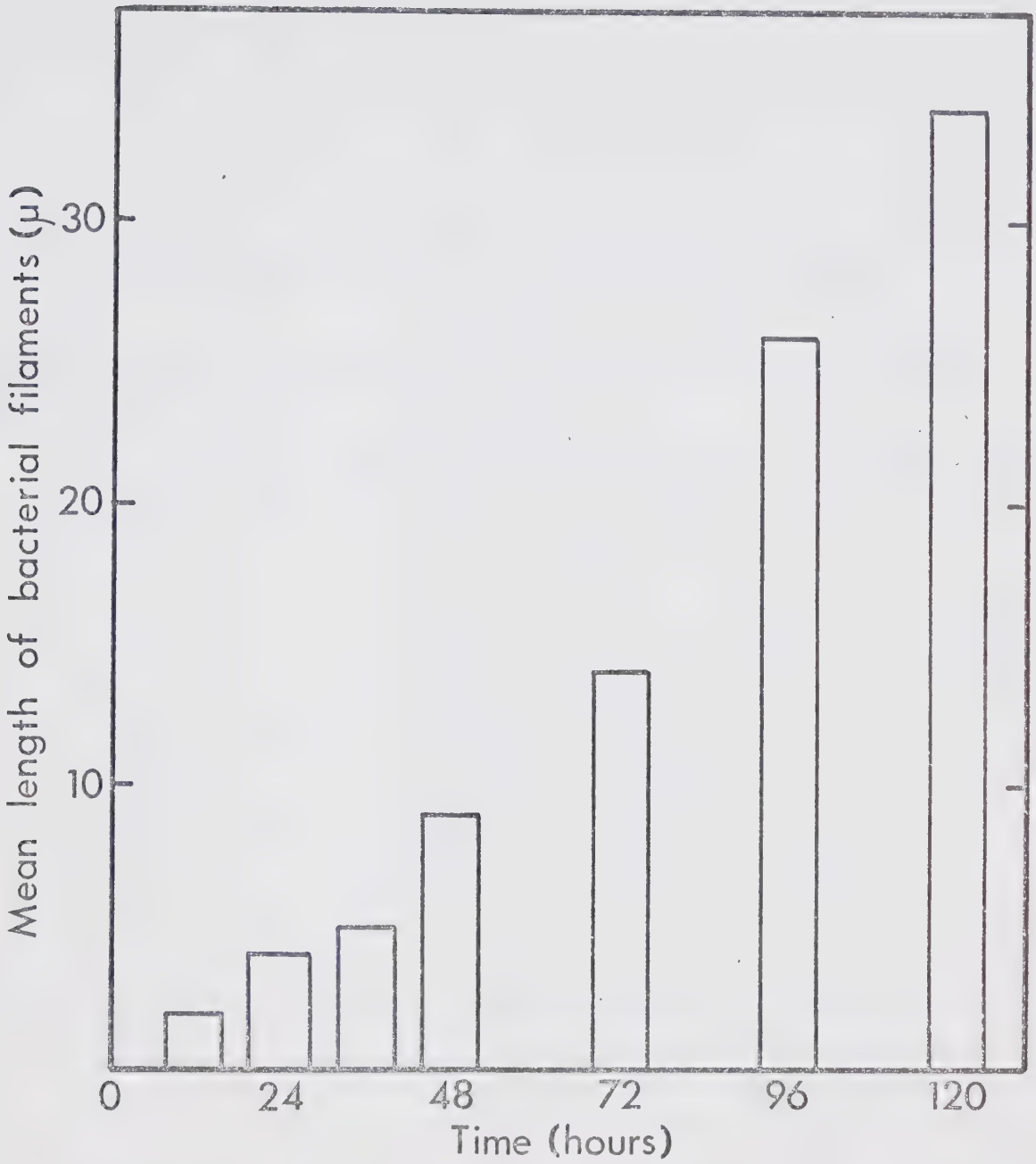


Fig. 9. Filament formation in S. typhimurium during growth at 10° C.

predominant. However, filamentation was more readily induced in minimal salts medium at 15° C than at 10° C in complex medium. From these observations on both minimal medium and complex medium, it is clear that morphological transformation in S. typhimurium is determined by incubation temperatures and enhanced by nutritional limitation of the culture.

Effect of Shift in Temperature from 10° C to 37° C on the Bacterial Filaments

The ability of the filaments to revert to normal rods was followed by determining the average cell length of 100 cells as a function of time after the cells were transferred from 10° C to 37° C. It can be seen quite clearly from Fig. 15 that the filaments were induced to revert to regular rods when transferred to the favorable temperature for growth. Within 4 hours after the temperature up-shift the filaments had reverted almost to their normal size. The photographs of stained preparations obtained at an hourly interval after temperature up-shift lends a strong support to this observation. Fig. 10(a) shows cells growing exponentially in a complex medium while Fig. 10(b) shows the filamentous growth between 3 and 4 days old. After the filaments were shifted to 37° C there was a gradual breakdown of the filaments as the incubation continued. This gradual fragmentation is demonstrated in Figs. 11 - 13. At the fourth hour, the filaments have reverted almost entirely to the normal length as shown in Fig. 13(b). From this evidence, filamentous growth resulting from low temperature incubation is reversible by

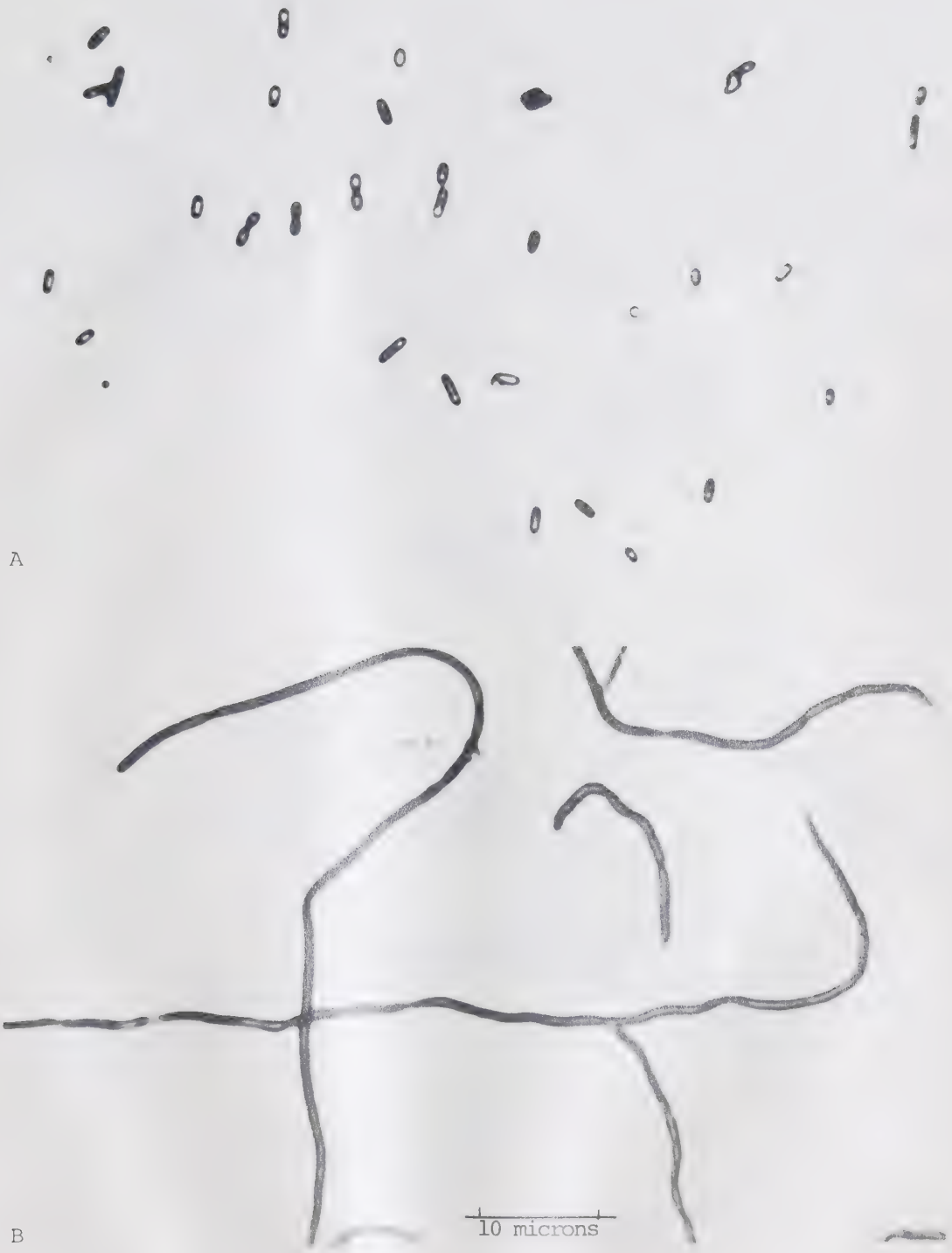


Fig. 10. (a) Cells of Salmonella typhimurium growing exponentially at 37° C in a complex medium (TSB); (b) filamentous growth of the same organism after four days incubation at 10° C in the same medium. Scale length = 10 μ .

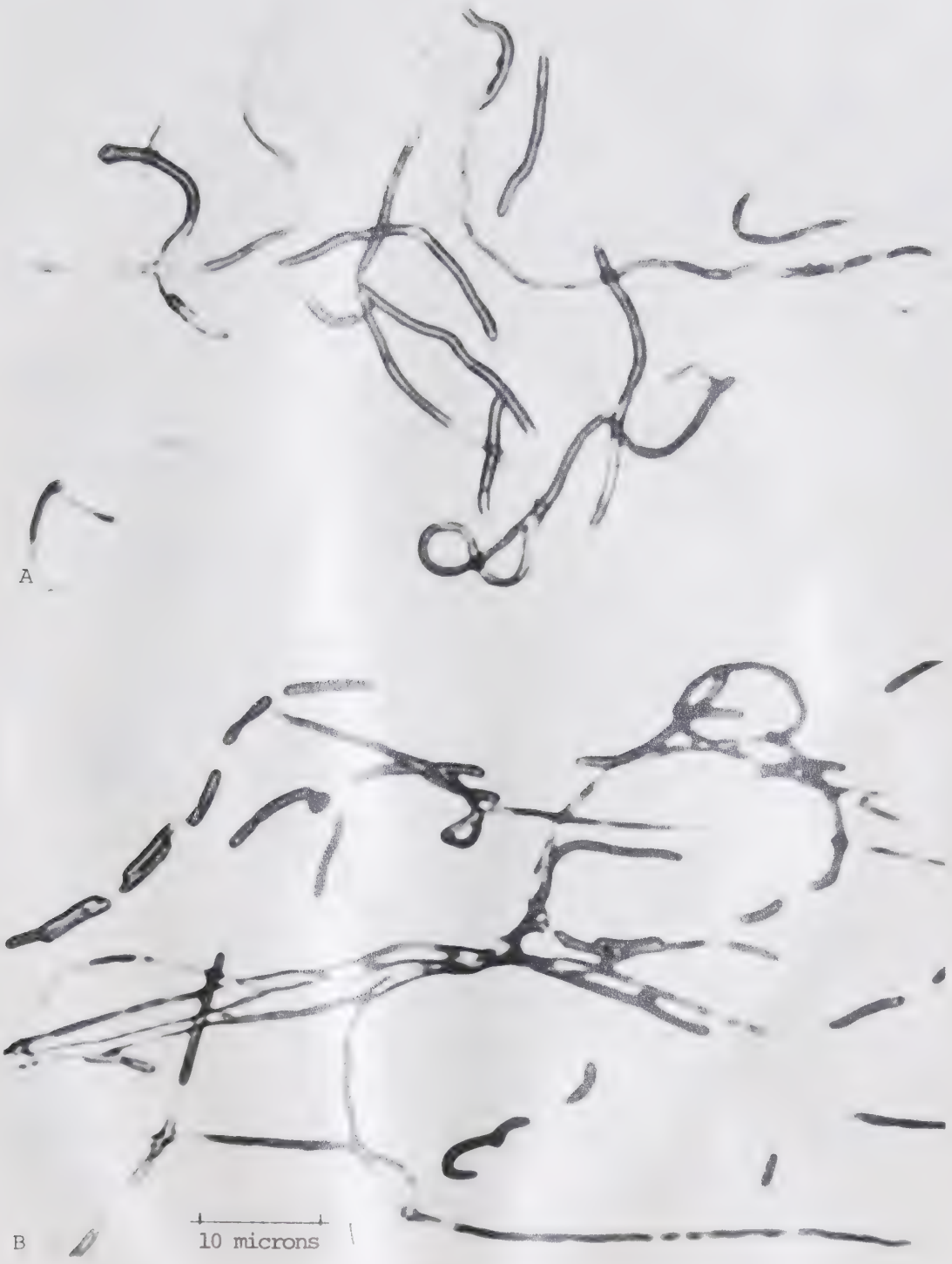


Fig. 11. (a) Cells of Salmonella typhimurium 30 minutes after shift of incubation temperature from 10° C to 37° C ; (b) 60 minutes after shift.

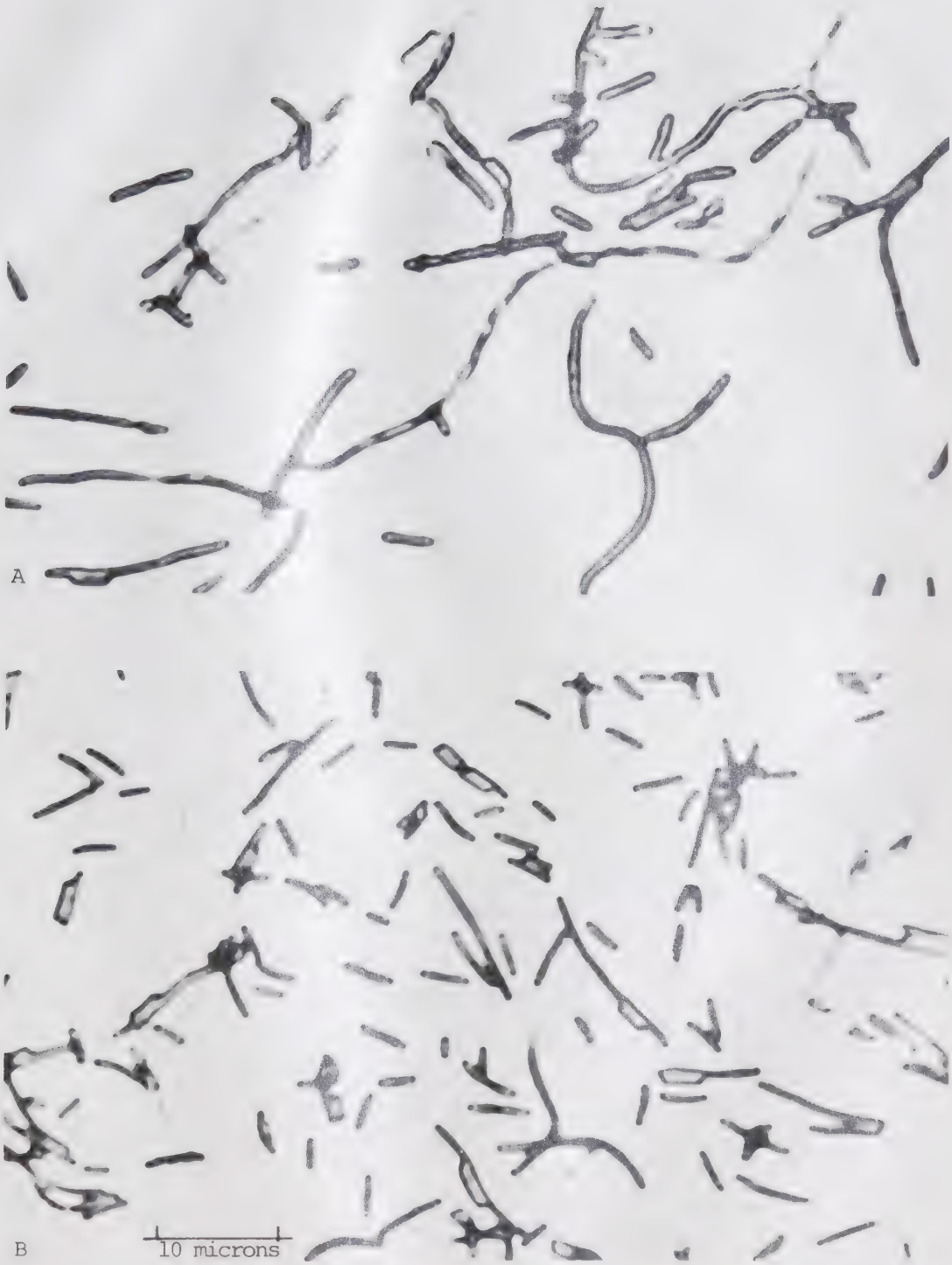


Fig. 12. (a) Cells of Salmonella typhimurium 90 minutes after shift of incubation temperature from 10° C to 37° C; (b) 120 minutes after shift.

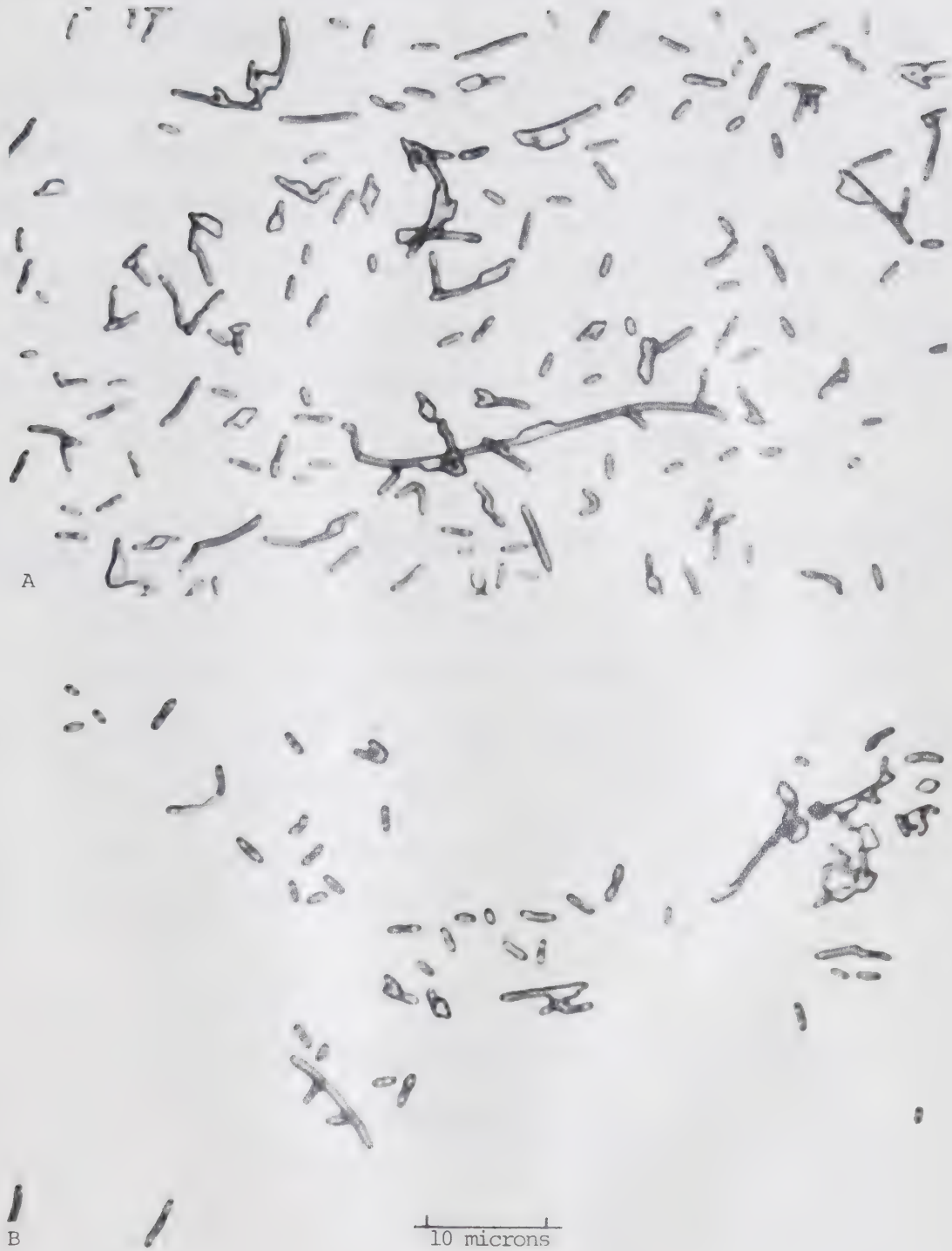


Fig. 13. (a) Cells of Salmonella typhimurium 180 minutes after shift of incubation temperature from 10° C to 37° C ; (b) 240 minutes after shift.

incubation at the optimum temperature for growth.

It was observed that when the filamentous culture growing at 10° C was transferred to the optimum temperature for growth, there was a rapid fragmentation of the filaments which slightly exceeded the rate of division of exponentially growing cultures at 37° C which have not been subjected to low temperatures. This is shown in Fig. 14. It was further noted that within 10 minutes after the shift in temperature, there was an obvious increase in colony forming cells.

Effect of Inhibitors on the Recovery of Filamentous Cells When Shifted from 10° C to 37° C

Since the filaments formed at 10° C were capable of reverting to normal rods when incubated at the favorable temperature for growth, an attempt was made to determine the lesion which prevents cell division. This was done by incorporating specific metabolic inhibitors in the medium during the temperature shift from 10° C to 37° C.

A. Inhibition of Cell Wall Synthesis

Penicillin has been shown to have two distinct morphological effects on Escherichia coli. One is the inhibition of cell division at low pencillin concentration and the formation of spheroplasts at high concentration. In general, pencillin was shown to interfere with the synthesis of the peptidoglycan of the bacterial cell wall. Izaki et al. (1966) have characterized two enzyme systems involved in the last steps in a sequence of reactions required for cell wall synthesis that are sensitive to low concentration of

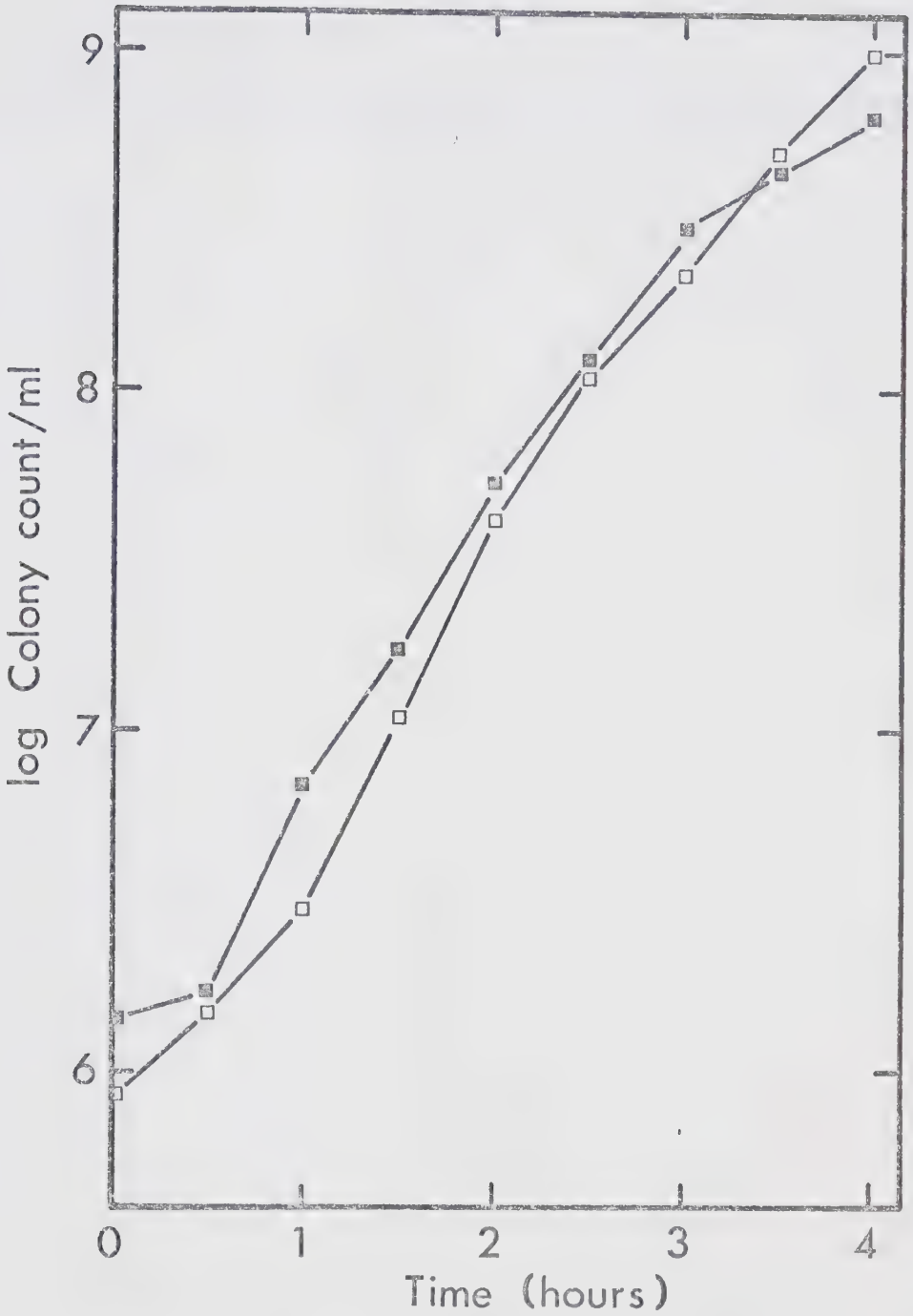


Fig. 14. Increase in colony count of filamentous culture after shift to 37° C compared to an exponential culture grown at 37° C. ■- Exponentially growing culture □-Filamentous culture.

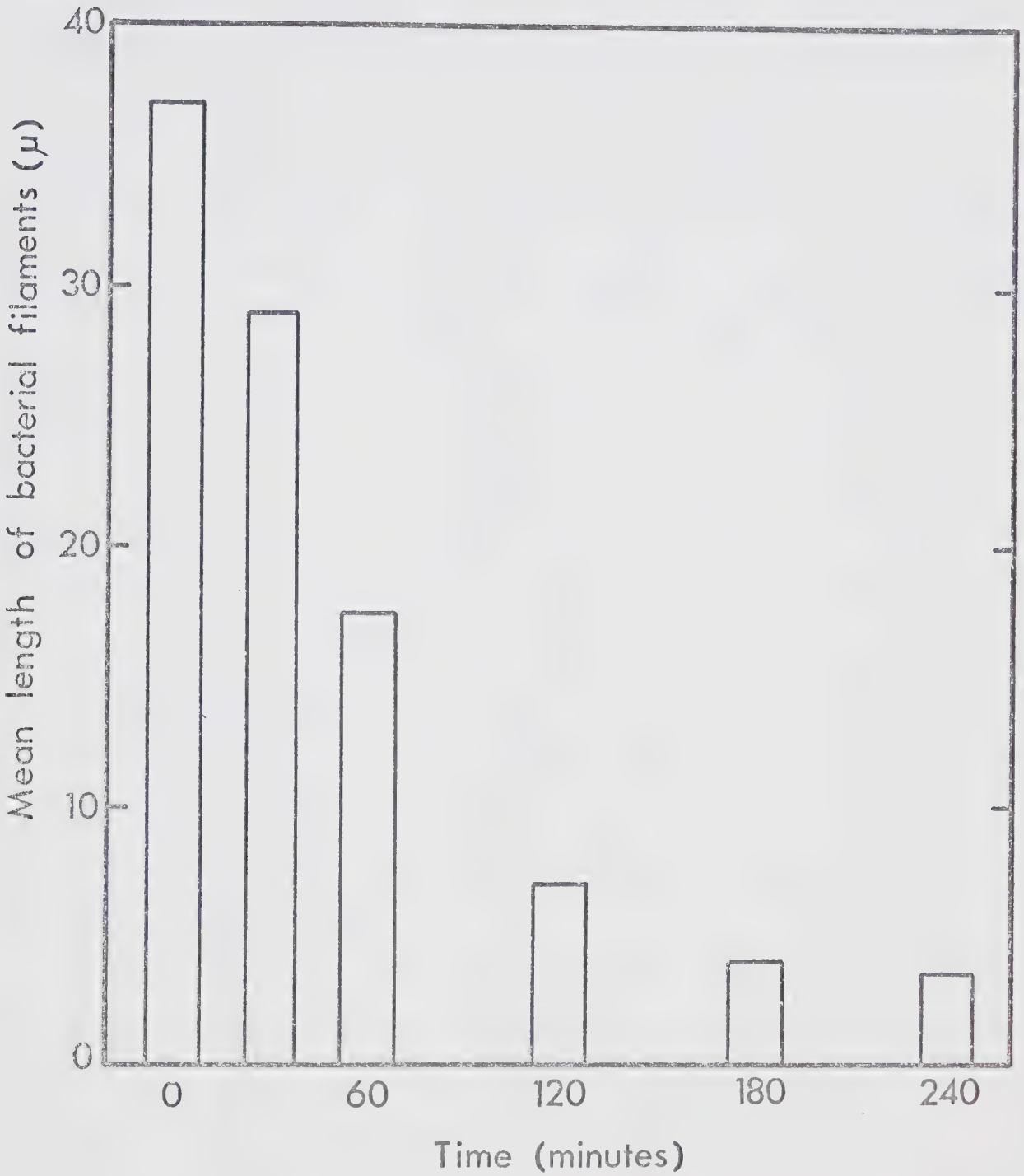


Fig. 15. Reversion of filamentous growth after shift in temperature from 10° C to 37° C.

pencillin. For D—alanine carboxypeptidase a concentration of 0.04 µg/ml of penicillin G was sufficient to cause 50% inhibition of activity while for glycopeptide transpeptidase a concentration of 3 µg/ml was sufficient to inhibit 50% activity. A concentration of 30 µg/ml was sufficient to inhibit growth of bacteria. Schwarz et al. (1969) and Hartman et al. (1972) found that 10 — 50 units/ml penicillin specifically inhibited cell division without affecting longitudinal cell growth and a concentration of 500 units/ml or more led to spheroplast formation. In this experiment the concentrations of penicillin G used were 10 µg/ml (16.5 units/ml) and 0.04 µg/ml (0.06 units/ml). From the data, it is seen that the filaments are very sensitive to penicillin as the addition of this antibiotic to the recovery medium at 10 µg/ml resulted in a marked decrease in viability of the cells. However, at a concentration of 0.04 µg/ml which caused 50% inhibition of D—alanine carboxypeptidase (Izaki et al., 1966) the recovery of the filaments and their ability to form colonies was not hampered as shown in Fig. 16.

D—cycloserine has essentially the same effect as penicillin on bacterial cell wall synthesis. Strominger et al. (1960) have shown that this antibiotic interferes in the racemization of L—alanine to D—alanine the required form for murein polymer and likewise, it was shown to interfere with D—alanyl - D—alanine peptide formation by D-alanyl - D—alanine synthetase in Streptococcus faecalis (Neuhaus, 1962) thus inhibiting the accumulation of the

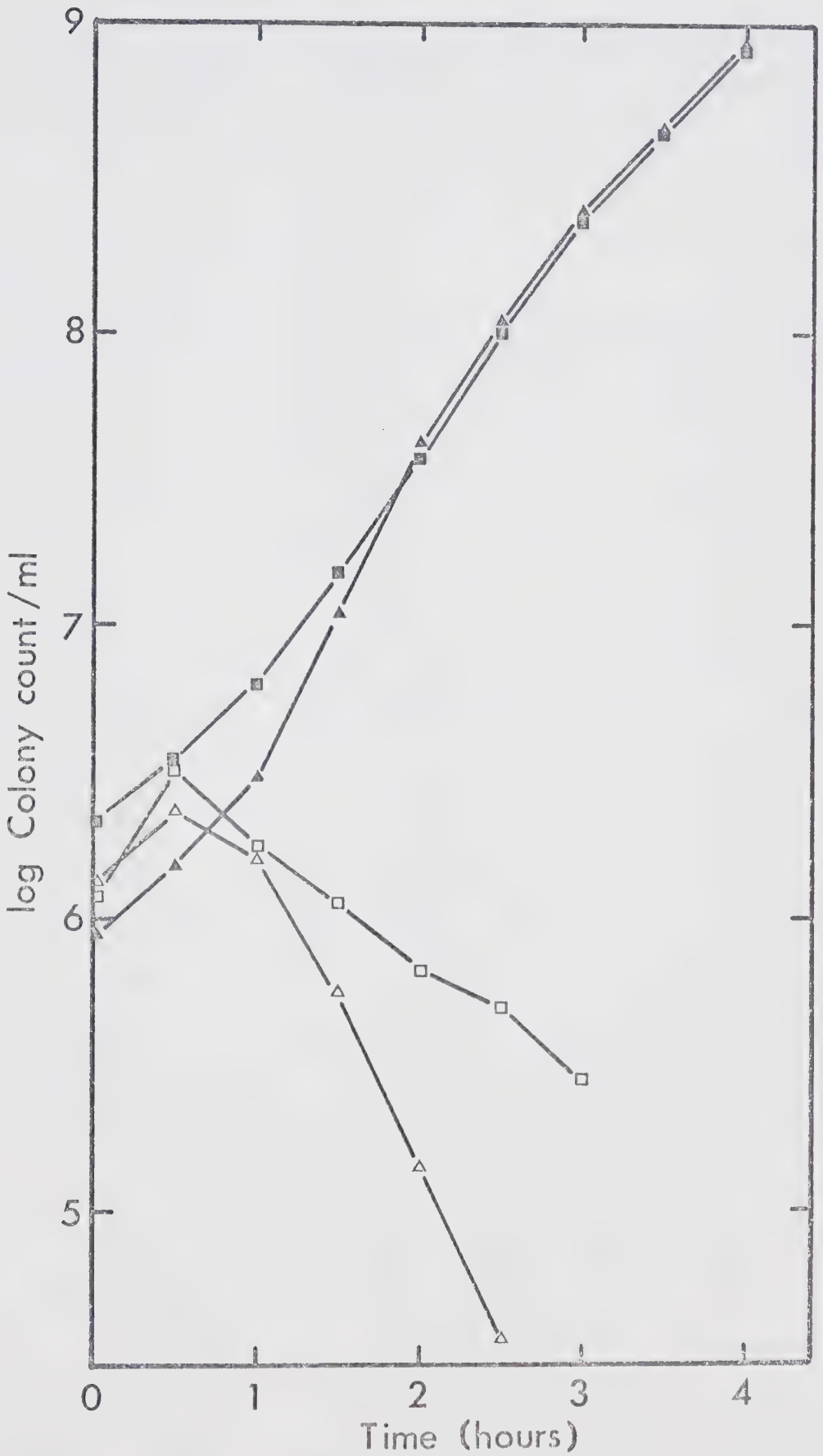


Fig. 16. Effect of cell wall inhibitors on the recovery of filamentous cells when shifted from 10° C to 37° C. Penicillin added: △ - 10 µg/ml; ▣-0.04 µg/ml; ▲ - no pencillin □ - 50 µg/ml D- cycloserine.

necessary nucleotide precursor for peptidoglycan synthesis. The addition of 50 µg/ml of this antibiotic in the recovery medium gave a similar result to penicillin as shown in Fig. 16.

B. Inhibitors of DNA synthesis

In view of the suggested role of DNA in cell division an attempt was made to find out if inhibition of DNA synthesis during cell recovery from the adverse effect of low temperature would prevent the filaments from undergoing cell division. Nalidixic acid is found primarily to inhibit DNA synthesis in susceptible organisms (Goss et al. 1965) particularly gram negative rods, while protein and RNA synthesis continue almost unaffected for some time after exposure to the drug. If the DNA formed by the filaments during growth at 10° C is normal and the segregation precise the inhibition of DNA synthesis at the time of shift to 37° C should not prevent division from the start of the shift. This can be determined by the addition of inhibitor of DNA synthesis. Results of the effect of addition of 10 µg/ml of nalidixic acid on the recovery of the filaments when the culture is transferred back to 37° C are given in Fig. 17. It shows that there is an increase in colony count which is evidence that cell division occurred for about 30 minutes after addition of the drug and after such time, the viability of the filaments rapidly declined.

C. Inhibition of protein synthesis

When chloramphenicol is added to bacterial cultures

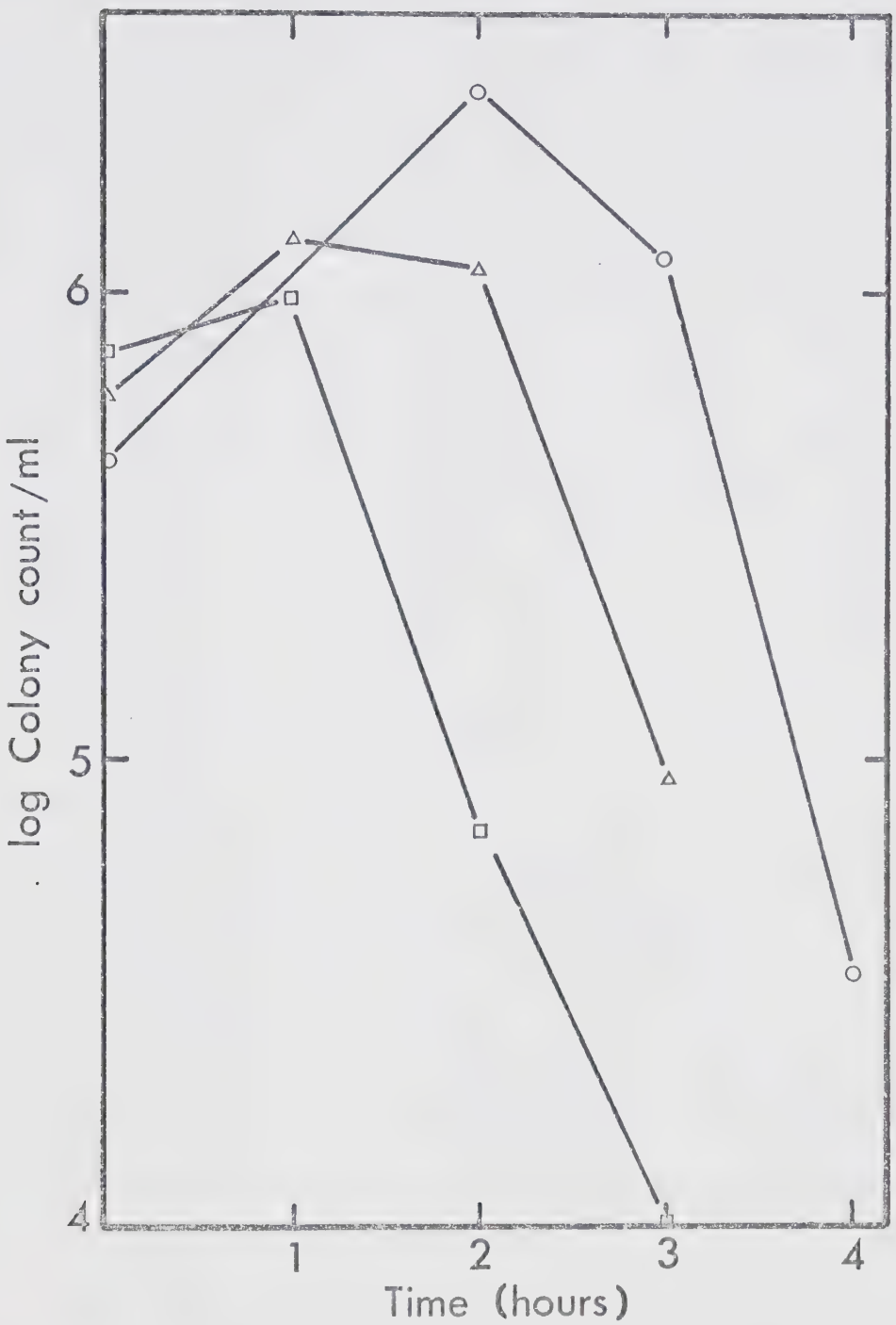


Fig. 17. Effect of Nalidixic acid on the recovery of filamentous cells during shift from 10° C to 37° C. 10 µg/ml nalidixic acid was added at □ - 0 time; △ - 10 minutes and ○ - 30 minutes after shift.

at growth inhibitory concentrations, (Vasquez, 1966) protein synthesis stops but the process involved in the concentration of small molecules within the cell is not affected. This antibiotic was found to inhibit preferentially the synthesis of inducible enzymes (Nakada and Magasanik, 1964) as a secondary effect due to catabolite repression. It has been found that this antibiotic does not inhibit either the activation of amino acids or formation of amino-acyl – S – RNA but inhibits the synthesis of protein by blocking the growth of peptide on the ribosomes (Julian, 1965). However, it does not affect the activity of the preformed proteins or enzymes and neither does it affect the synthesis of cell walls (Vasquez, 1966).

Chloramphenicol was added to the recovery medium to determine if protein synthesis is required for the recovery of the cells at 37° C. Since chloramphenicol inhibits the synthesis of polypeptide chains or synthesis of proteins but does not affect the activity of the preformed proteins or enzymes, the filaments would be able to proceed to recovery even in the presence of chloramphenicol if proteins necessary for the recovery were preformed at the low temperature prior to the shift to higher temperature.

The requirement for protein synthesis during the recovery period wherein chloramphenicol was added to the individual cultures at the time of temperature shift and 30 minutes and 60 minutes after shift, is shown in Fig. 18. Contrary to the observation of Reeve et al. (1970) wherein filaments formed at 42° C were capable of dividing at the

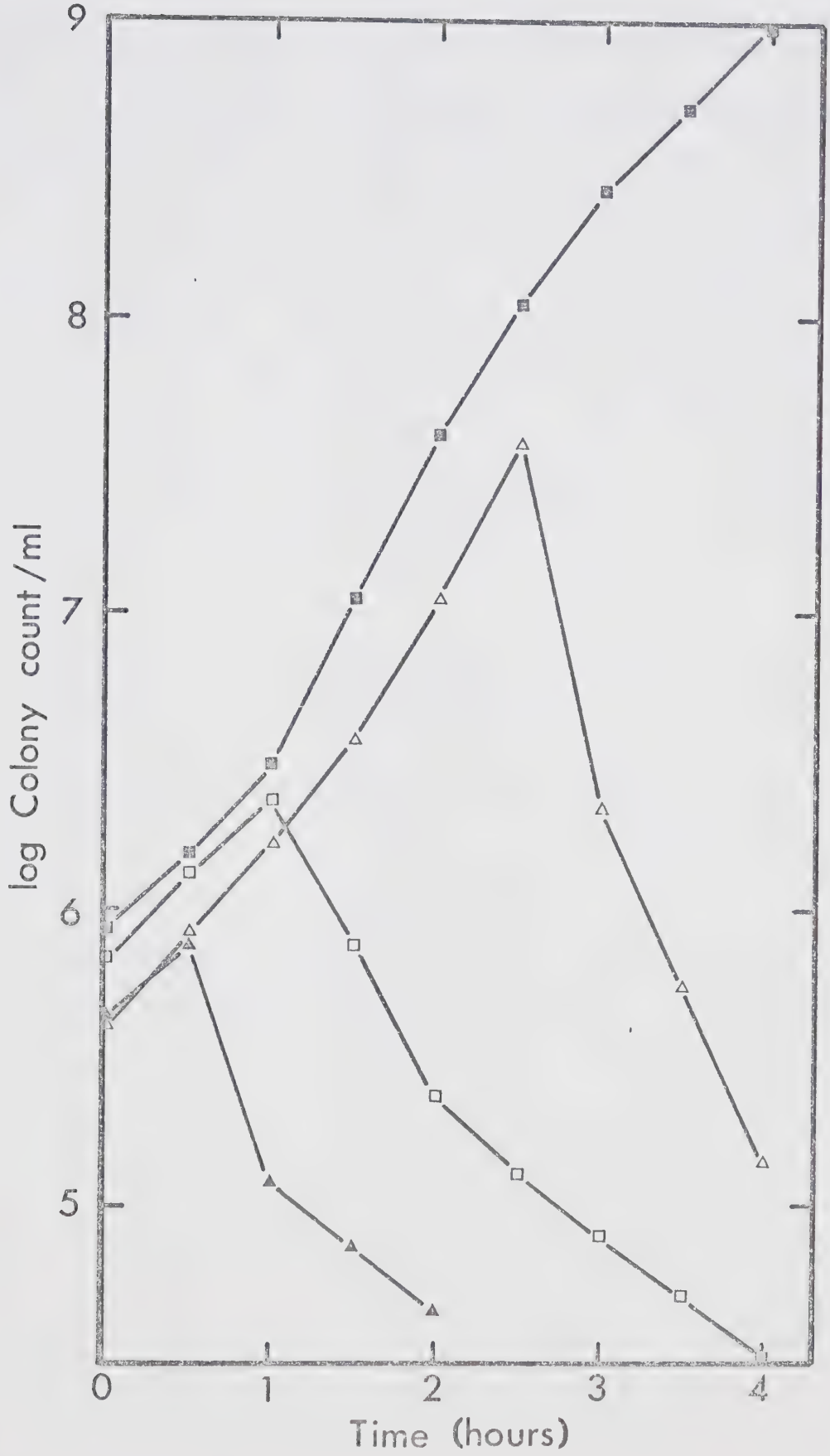


Fig. 18. Effect of chloramphenicol on the recovery of filamentous cells during shift from 10° C to 37° C. Chloramphenicol (10 µg/ml) added: ▲ - 0 time; □ - 30 minutes; △- 60 minutes after shift and ■- no chloramphenicol.

optimum temperature in the presence of 150 µg/ml chloramphenicol , in the present experiment, the presence of 10 µg/ml chloramphenicol allowed residual cell division for only a short period relative to the addition of the drug during recovery and that after this period, the cells progressively lost their viability. This observation is rather similar to that of Spratt and Rowbury (1970) in their experiment with Salmonella induced to form filaments at 42° C. The strain that they used however required chloramphenicol concentration 20 times greater than that required to bring the same effect as the bacterial strain used in this experiment.

Influence of Cell Free Extract on Cell Division at 10° C

In one aspect of the experiment where the inoculum reached the threshold of stationary phase before it was transferred to 10° C, it was observed that filamentation was not as pronounced as observed in previous batches and that most of the filaments appeared as chains rather than as continuous strands. Due to the relatively high level of inculum used (50% v/v) it was assumed that the cell division factor which has been synthesized by the cells in the inoculum at 37° C, became available to the new cells after they have been released by autolysis. An attempt was therefore made to determine the effect of the presence of cell free extract on the ability of the cells to divide. Cell free extracts were prepared as described in Materials and Methods. Three flasks were prepared as follows: (1) medium plus cell extract (1 ml/100 mls medium; (2) medium plus cell extract (1 ml/100 mls), the whole contents then heated at 100° C

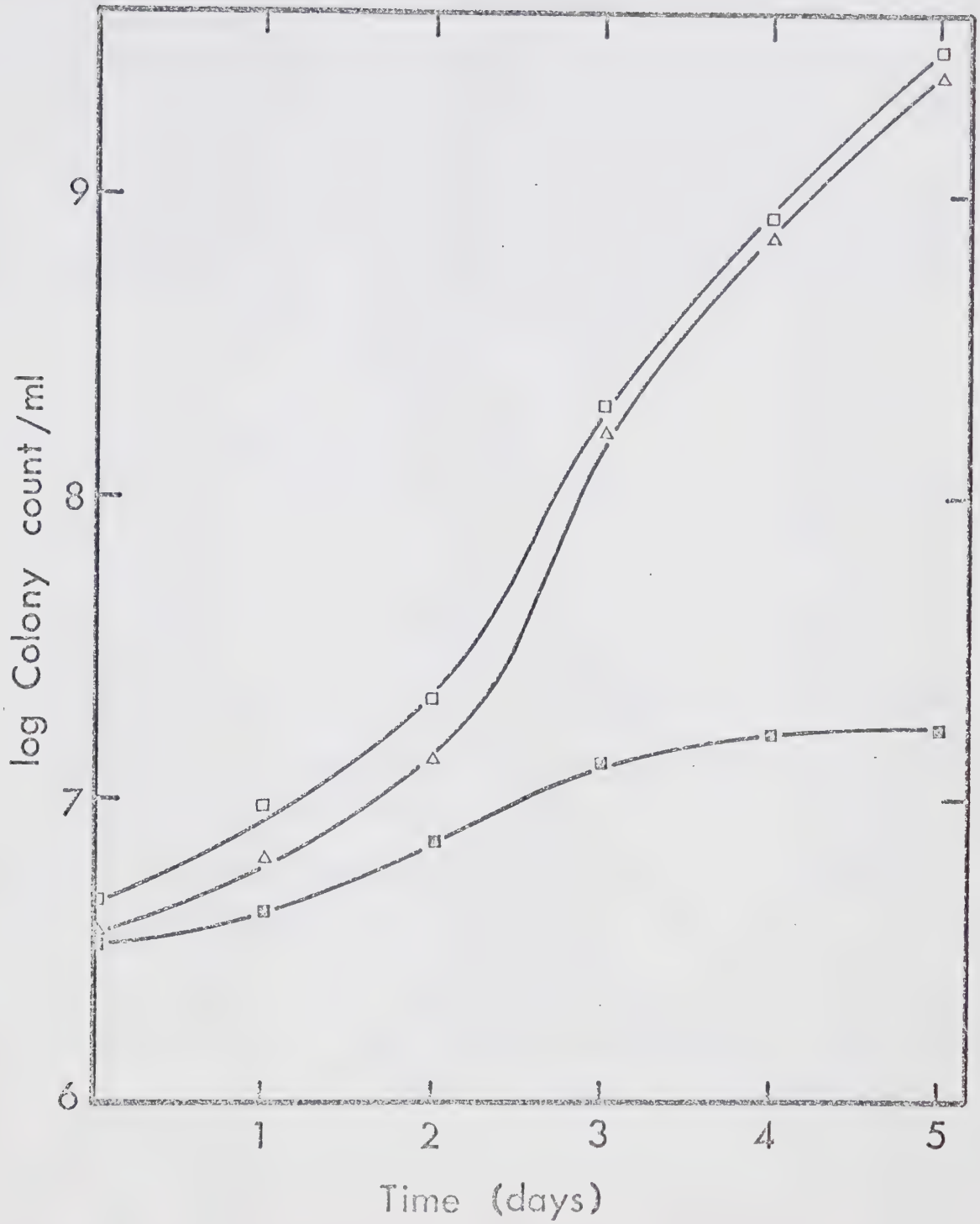


Fig. 19. Effect of cell extract on the growth and division of S. typhimurium during incubation at 10° C. □- heated cell extract added; Δ- unheated cell extract added; ▣- no cell extract added.

for 10 minutes; (3) medium only. Following inoculation the growth of the cultures was monitored and the results are shown in Fig. 19. It is quite clear that the addition of cell extract induced cell division at 10° C as shown by the differential increase in colony counts over that of the control. One interesting point however is that whether the cell extract was heated or unheated, it resulted in similar induction of cell division.

Electron Microscopy

Electron micrographs were prepared with the purpose of determining if there were any differences in the intracellular morphological organization when the cells were grown at the two different temperatures. Fig. 20 shows the normally growing cells at 37° C. The cells undergoing cell division show a well separated nuclear material and cell wall constriction along the length of the cell. It seems that incubation at 10° C does not result in any gross alteration of DNA synthesis and nuclear division as it is shown in Fig. 21 that the nuclear division appears to proceed normally in the filamentous cell. This evidence would support the finding that similar to growth at 37° C increase in DNA proceeds in the normal pattern during growth at 10° C.

One point of interest however is shown in Fig. 22 where it can be seen that the walls of the cells grown at 10° C appear to be loosely attached to the cytoplasmic membrane. This is in contrast with the cell walls of the cells grown at 37° C in which the walls are tightly bound

Fig. 20. Cross-section of cells of Salmonella typhimurium grown at 37° C in Trypticase Soy Broth. Cells undergoing cell division show a well separated nuclear material (N) and a constriction of the cell wall at the site of cell division (D). Magnification, 27,000

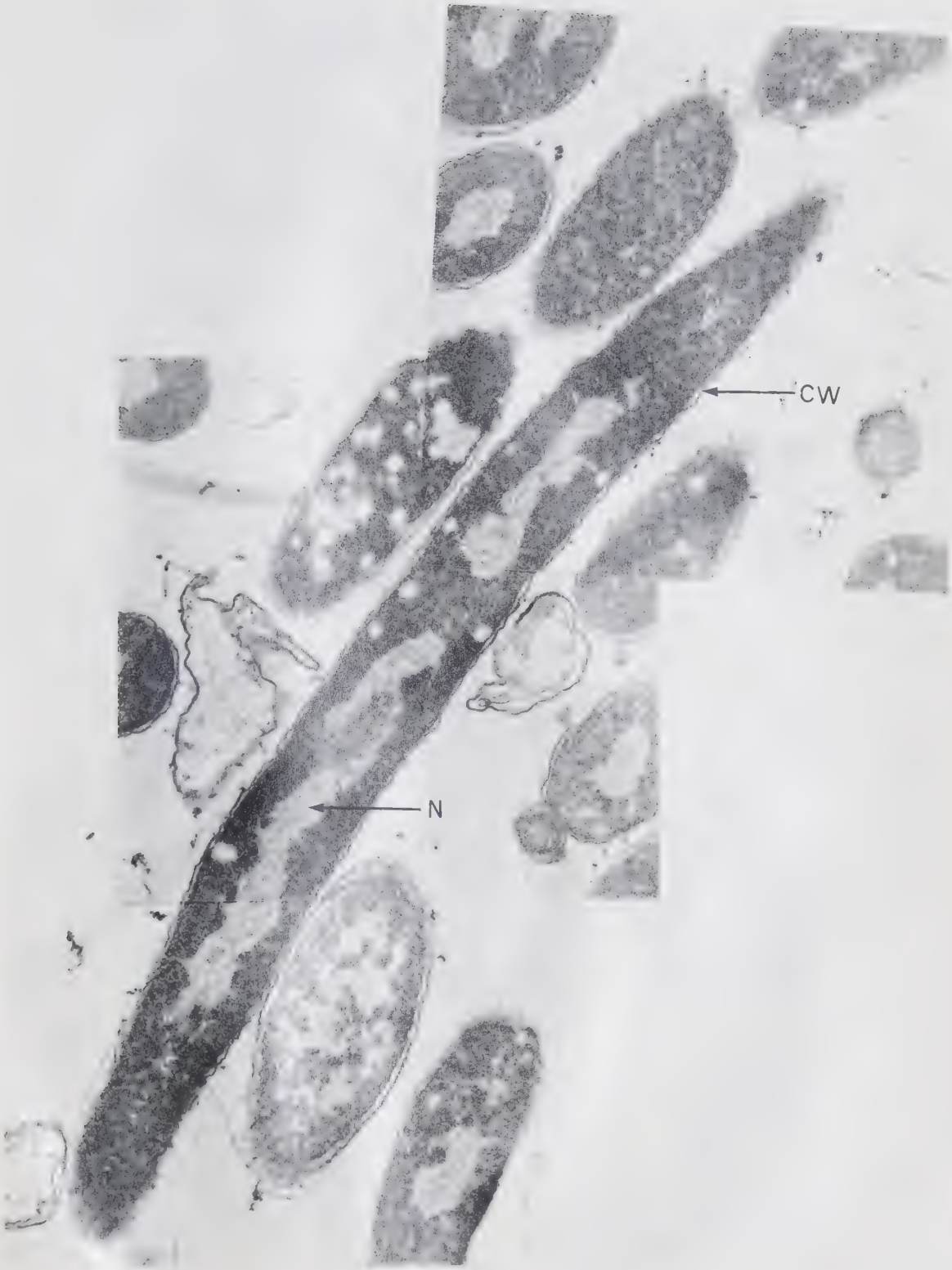


Fig. 21. Cross-section of cells of Salmonella typhimurium grown at 10°C in Trypticase Soy Broth. Nuclear material (N); Cell wall (CW) Magnification, 55,000

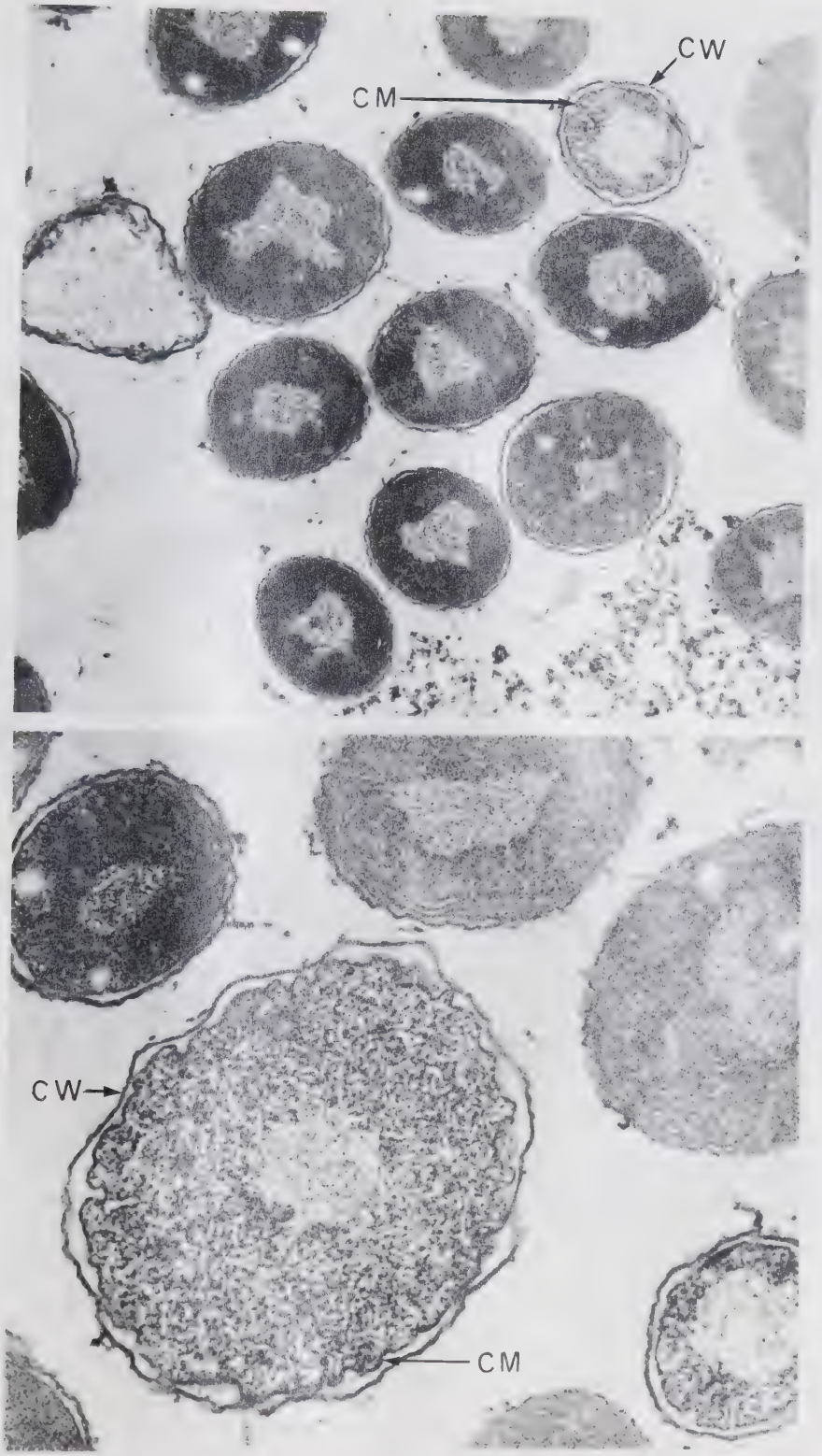


Fig. 22. Cross-section of cells of Salmonella typhimurium grown at 10° C in Trypticase Soy Broth. Cell wall (CW); cytoplasmic membrane (CM). Magnification, 43,500

Fig. 23. Cross-section of cells of Salmonella typhimurium grown at 37° C in Trypticase Soy Broth. Cell walls (CW); cytoplasmic membrane (CM). Magnification, 55,000

to the cytoplasmic membrane as shown in Fig. 23. This morphological defect which appears as a loose binding of the cell wall to the cytoplasm produces a gap which appears similar to a periplasmic space produced in E. coli which Wetzel et al. (1970) attributed to the shrinkage of the protoplast by hypertonic solutions.

DISCUSSION AND CONCLUSIONS

The present study was primarily undertaken to determine the effects of sub-optimal incubation temperatures on the growth of S. typhimurium. In the course of the work however, it was noted that the cells formed filaments at 10° C. In the view of this, most of the subsequent studies were aimed at the elucidation of the nature of the filaments and factors affecting their formation and fragmentation.

The growth curves for cultures incubated at 10, 15, 20, 25 and 37° C are expected with the increases in growth occurring with increasing temperature. The optimum growth temperature of S. typhimurium is generally reported to be 37° C. One noticeable feature however is the long lag phase that occurs at 10° C. Another peculiarity of cells grown at 10° C is that they do not exhibit a linear relationship between cell mass and colony count as measured by absorbance and plate count respectively.

Microscopic examination revealed that during incubation at 10° C the cells failed to undergo cell division and as a result long filaments are produced. Since septum formation is a prerequisite for bacterial reproduction this would explain the data that cell number does not increase proportionately with cell mass because the filaments fail to form daughter cells that are capable of forming colonies. Robinow (1944) stated that during balanced growth, bacteria

divide at constant intervals and at each division cycle, the nuclei occurring singly or in pairs are replicated, the cell elongates and the DNA replicas progressively separate from each other. A septum is then formed near the equatorial plane of the cell so that two cells, each one containing half of the DNA content of the mother cell are formed and separate from each other.

The results on macromolecular composition of the organism grown at 10° and 37° C do not reveal any major differences that might account for the formation of filaments. However it is possible that qualitative changes in one or more of the components could be responsible for this phenomenon.

What mechanism then, controls septum formation whose function is impaired at low incubation temperature? There is a general agreement that a causal relationship exists between DNA replication and cell division. This comes from the observation that agents that specifically inhibit DNA synthesis such as U.V. irradiation (Adler and Hardigree, 1964); thymine starvation (Cohen and Barner, 1954); high temperature (Walker and Pardee, 1967) often resulted in the failure of the cells to undergo cell division process and therefore eventually form filaments devoid of septa or cross-walls. On the other hand, Smith and Pardee (1970) observed the requirement for the accumulation of a protein for cell division during the cell cycle of E. coli and that inactivation of this component by heating above the optimum temperature resulted in filament formation. Furthermore,

Starka and Moravava (1967) observed filamentation in E. coli treated with penicillin which is generally known to interfere in the synthesis of bacterial peptidoglycan. Goss et al. (1965) have demonstrated that the formation of elongated serpentine forms of E. coli treated with nalidixic acid is an effect of the inhibition of DNA synthesis.

A detailed study of growth and division at 10°C has shown that growth is characterized by a lag phase of approximately 24 hours. Microscopic observations indicate that the cells undergo a lag phase characterized by the absence of cell division. At approximately 24 hours however, there appears to be a burst of cell division as shown by the general appearance of constrictions along the length of the cells. After one round of cell division the cells are no longer able to divide but rather, cells increased in mass without undergoing cell division, thus resulting in the formation of long filaments with continued incubation, the length of the filaments extending to as much as a hundred times that of a normal cell. From these observations therefore, it can be postulated that the cell division factor is preformed at 37°C prior to the shift in temperature to 10°C and that due to the slow growth rate at 10°C, there is a delay in cell division for approximately 24 hours. At 10°C, either the activity or the synthesis of this cell division factor is inhibited, and a higher temperature is required for this factor to either start synthesis or regain its activity as evidenced by the fragmentation of filaments that occurs on

transfer back to 37° C. That this factor is more directly influenced by the temperature rather than the nutritional composition of the environment is based on the observation that the same medium was used in the shift experiments and furthermore, whether the organism was grown in complex medium (TSB) or minimal medium, filamentation similarly appeared at low incubation temperature. As stated in the results, observations in complex medium at 8° C also showed filamentous cells but the length of the filaments is shorter than those formed at 10° C. This is probably due to the faster growth at 10° C and longer filaments would be obtained proportional to the increase in cell mass. Observations at 12° C in the same medium showed a few filamentous cells which upon prolonged incubation form into normal rods. It would seem therefore that at 12° C, the cell division factor is synthesized and active to a limited extent or that after the cells are given time to allow to adapt to the new temperature, the activity of the cell division factor is maintained.

This filamentation resulting from such low temperature incubation is similar to that obtained with heat sensitive mutants of E. coli (Reeve et al., 1970; Van de Putte et al., 1964; Inouye, 1969); B. subtilis (Donachie et al., 1971) and S. typhimurium 4a and 11G (Ahmed and Rowbury, 1971). One common response of these organisms however in contrast with that of the present experiment is that, this filamentation was exhibited when these were incubated at temperatures higher than their optimum temperature for growth. However,

in common with the present experiment, they all show the ability of the filaments to regain their normal size when transferred to the optimum temperature for growth. Hence they have been aptly called conditional thermosensitive mutants (Hirota et al., 1968). Two general effects were observed in these microorganisms which caused filamentation at high temperature of incubation. Van de Putte et al. (1964); Walker and Pardee (1967) and Spratt and Rowbury (1970) observed that their organisms were sensitive for DNA synthesis at 42° C while Donachie et al., (1971) and Ahmed and Rowbury (1971) observed that incubation at 42°C destroyed a protein which had to be resynthesized before cell division could restart.

As mentioned earlier, transferring the incubation temperature from 10° C to 37° C resulted in the recovery of the filamentous cells into normal rods. As shown in the photographs in Figs. 10 - 13, at 30 minutes after the temperature shift there was already a noticeable fragmentation of the filaments and the division continued until almost a normal size was attained within the first four hours. Constrictions appeared at various points throughout the filaments. It seems possible that the filaments could undergo cell division very rapidly after a shift in temperature as Pardee and Prestige (1961) have shown in their studies on the kinetics of induction of inducible enzymes. They found that β - galactosidase, D-serine deaminase, aspartate transcarbamylase, tryptophanase and thymidine phosphorylase showed

a lag of 3 minutes between addition of inducer and appearance of enzyme and that the duration of the lag was temperature dependent. If in this present experiment the inhibition of cell division was due to the requirement for a particular enzyme, at say, near the end of the cell cycle, then it is reasonable that cell division could occur within 10 minutes after a shift in temperature.

The results of the experiments with metabolic inhibitors shed some light on the phenomenon of filamentation. Thus both penicillin and D - cycloserine fail to prevent an increase in colony count during the first 30 minutes after filaments are transferred from 10° C to 37° C. A similar cultural response is seen with nalidixic acid. From these results it can be concluded that the cell wall synthesis and DNA synthesis that occur at 10° C is normal. Although nalidixic acid inhibits cell division due to a primary effect on DNA synthesis, cells which have completed rounds of DNA synthesis prior to the addition of the inhibitor divide apparently normally (Hemstetter and Pierucci, 1968).

Similar increase in colony counts were observed in the presence of chloramphenicol after transfer on incubation temperature. This response exhibited by this organism is in contrast with that of E. coli (Reeve et al., 1970) and S. typhimurium 4a (Ahmed and Rowbury, 1971) which showed either a long lag phase or no recovery when the cultures were transferred to the favorable temperature in the presence of the same inhibitors. The speed at which the cells undergo cell

division would mean therefore that with the organism in the present experiment, the factor necessary for the formation of the septum and the separation of the cells are indeed made at low temperature but either its activity is blocked or some assembly process can not take place at the low temperature whereas in the work of Reeve et al. (1970) and Ahmed and Rowbury (1971) the cell division factor is destroyed at 42°C which perhaps is the reason for the lag, at which time the cells have to restart the synthesis of this factor.

It is clearly shown that the extract from a stationary culture of the same organism grown at 37°C is capable of reversing the impairment of cell division in S. typhimurium induced by incubation at 10°C. Karganonkar and Raut (1967) used similar extract in an attempt to induce cell division in U.V. irradiated cell and he assumed that the factor that was responsible for the induction of cell division was a nucleoprotein due to the inhibition of its activity by heating at 52°C. Mukhergee and Bhattacharjee (1970) similarly believed that the active component was exclusively a protein on similar grounds. In the present experiment however, it seems unlikely that a protein component alone is principally affected or involved since heating the cell extract at 100°C for 10 minutes did not cause a decrease in activity as shown by the similar increase in colony counts with a culture containing unheated extracts. Adler et al. (1966) in using similar extracts to induce cell division in irradiated E. coli which grew into

filaments have shown that the system responsible for promoting cell division was relatively complex involving both heat labile and heat stable compound. These authors later (1969) found that this factor was not inactivated by pronase, trypsin, chymotrypsin, ribonuclease, deoxyribonuclease, lysozyme or α-amylase but phospholipase A completely inactivated the division promoting activity and detergents likewise had some inhibitory effect. Phospholipase A has been shown by Van Deenan and de Haas (1963) to attack phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol and phosphatidic acid. Furthermore, lipases substantially decreased their division promoting activity. The results of the present study are not extensive enough to compare with those of Adler et al., but it is interesting to note that the factor that promoted cell division at low temperature was heat stable. Some lipids are essential membrane components which play an important role in the biosynthesis of lipopolysaccharide and peptidoglycan. The active lipid such as phosphatidylethanolamine acts as a physical co-factor but not itself participating in the biosynthetic reaction sequence while the carrier lipid (polyisoprenoid alcohol phosphate) does participate directly in the biosynthetic sequence (Rothfield and Romeo, 1971). It has been suggested that the formation of a lipid intermediate is necessary for the mucopeptide precursors to cross the cell membrane to the external site of polymerization in the cell wall (Higashi et al., 1967). As shown by Dietrich et al.

(1967), peptidoglycan synthesis in Micrococcus lysodeikticus is catalyzed by a particulate fraction assumed to be derived from the bacterial membrane, containing both enzyme protein and essential phospholipid which act as carrier of the sugar fragments during peptidoglycan synthesis. They have further observed an obligatory involvement of the lipid fraction in the biosynthesis of the peptidoglycan since after separation of the two components, protein alone was inactive but the activity could be restored by recombination with the lipid. As mentioned in the introduction, Okuyama (1969) found that the synthesis of phosphatidylethanolamine was lower at 10°C in E. coli while at the same time, its degradation was enhanced. From the foregoing reports, and in view of the heat stability of the cell extract in this work it is possible that a lipid is indeed involved and that a protein is an ancillary component of a cell division factor.

As stated earlier, electron micrographs of cross-sections of cells grown at 37°C and 10°C were taken in order to see if structural features of the two types of cells were different. It is apparent that the nuclear material undergoes an orderly formation and segregation as shown by the presence of dense material that is regularly spaced along the filaments. However, no constrictions at the distance between the separated nuclear material appear. However, there is a change in the attachment of the cell wall to the cell membrane in the cells incubated at 10°C. Such alteration is characterized by the appearance of a periplasmic space.

Although the appearance of a periplasmic space in microorganisms is attributed to the effect of hypertonicity of the environment, it might also be the effect of what Grula (1972) observed as the leakage of proteins with presumed enzymatic activity from the outside peripheral area of the filamentous cells. The stability of the membrane integrity is perhaps then a very important factor that enables the whole cell to carry out its functions particularly cell division in a normal and orderly fashion. Jacob et al. (1963) postulated that the bacterial chromosome is attached to the cytoplasmic membrane and that the association is an important factor which enables the bacteria to undergo an orderly process of cell division. Such postulated association has been proven by cytological studies (Jacob et al., 1966 and Ganesan and Lederberg, 1965). That these observations from the electron micrographs is an effect of temperature rather than by the manipulation of the cells during preparation is evidenced by the fact that cells grown at 37°C do not show similar derangement in spite of the same treatment.

This investigation has shown that incubation of Salmonella typhimurium ATCC 13311 at 10°C brings about inhibition of cell division resulting in filament formation. The length of the filaments varies, reaching one hundred times that of a normal rod. The mechanisms that control the process of cell division are far from clear. Further work should be directed toward identification of factors in the cell extract that have the ability to promote fragmentation

of filaments. In fact the filament produced at 10°C would appear to be an ideal system for studies on the nature of cell division in bacteria.

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